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THE RELATION BETWEEN FIXATION OF PENICILLIN SULPHUR AND PENICILLINASE ADAPTATION IN *B. CEREUS*.

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IT was recently found (Pollock, 1950) that cells of *Bacillus cereus* reacted to treatment with penicillin for 1 hr. at 0°, by rapidly forming penicillinase when subsequently incubated in a penicillin-free casein hydrolysate medium. During incubation the rate of penicillinase production was approximately constant, and depended upon the penicillin concentration used for pre-treatment up to 1·0 unit/ml., at which level maximal effect occurred. These facts suggested that the effect of the penicillin might be due to its interaction with some specific receptor within the cell to form a complex or compound functioning as some sort of specific catalyst whose concentration determined the rate of penicillin formation.

Pasynskii and Kastorskaya (1947) reported that very small quantities of penicillin (amounting to about 1000 molecules/cell) were absorbed by *Staphylococcus aureus* growing in penicillin broth. Later, Maass and Johnson (1949*a*) and Rowley, Cooper, Roberts and Lester Smith (1950) showed that minute quantities of S (corresponding to a penicillin fixation per cell of the same order as that reported by Pasynskii and Kastorskaya were irreversibly fixed by *Staph. aureus* cells when treated with a very low concentration ($< 0{\cdot}1$ unit/ml.) of S^{35}-labelled penicillin.

The work here reported was therefore undertaken in order to see whether penicillin S was similarly fixed by *B. cereus*; and if so, whether any correlation could be found between the extent of such fixation and the rate of penicillinase production—as might be expected were the two phenomena interrelated.

METHODS.

Organism: *B. cereus* NRRL 569.—Washed suspensions were grown in tryptic meat broth from spore inocula for 16 hr. at 35° on a bacteriological shaker.

Penicillin.—Ordinary sodium benzylpenicillin was obtained from Glaxo Ltd. Purified S^{35}-labelled penicillin containing 0·16 μc/unit and prepared by growing *Penicillium chrysogenum* in a synthetic medium containing radioactive sulphate was kindly given us by Dr. Lester Smith of Glaxo Laboratories. Benzylpenicillin

constituted over 85 per cent of the total penicillin in this radioactive product and non-penicillin radioactivity amounted to about 5 per cent of the total.

Bioassay of penicillin was carried out by measuring zones of growth inhibition of a sensitive strain of *Bacillus subtilis* around cylinders containing penicillin solutions on a 0·3 per cent Lab Lemco, 1 per cent peptone, 2 per cent agar base (containing about 10^5 *B. subtilis* spores/ml.) poured into large square plates (10 in. × 10 in.). Two concentrations of standard Penicillin G and of the unknown were tested in octuplicate on the same plate. The solutions were allowed to diffuse out into the medium at + 2° for 4 hr. before incubation (16 hr. at 35°) in order to increase the sensitivity of the assay. The mean values for inhibition zone diameters and thus the potency ratio, with limits of probable error, of the unknown to the standard were then calculated.

Penicillinase activities were assayed by the manometric method of Henry and Housewright (1947), and refer to the total enzyme (combined extra- and intracellular fractions) present in the culture.

Penicillinase production was followed in 0·8 per cent casein hydrolysate medium (CH) with M/600 $MgSO_4$ and M/50 phosphate buffer, pH 7·0, aerobically, at 35°, samples being removed at intervals and M/1200 oxine added to stop further enzyme production (as described by Pollock, 1950). The only modification was the addition of 1 per cent gelatin to the medium, since it had been shown that this stabilized the enzyme against possible inactivation before assay.

Radioactivity measurements were done with an end-window (1·9 mg./sq.cm.) Geiger-Müller Counter. Thick washed suspensions of cells for counting were suspended in water to about 50 mg./ml. About 0·2 ml. was added to 2 mm. deep, 15 mm. diameter rimmed polythene discs (previously weighed) so that the fluid was just level with the top of the rim, thus avoiding uneven deposition of cells. The samples were then frozen as rapidly as possible on dry ice, dried *in vacuo*, pressed down evenly on the discs with a metal plunger, re-weighed and counted. The average weight of dried cell samples was 10 mg. Corrections for self-absorption were made by means of a curve prepared by mixing 0·02 unit of radioactive penicillin with different weights of cells (ranging from 4·3 mg. to 13·6 mg.), freeze-drying and counting in a similar manner.

Penicilloic acid, from radioactive penicillin, was prepared by bringing a solution of 50 units/ml. to pH 12, heating at 100° for 30 min., leaving at room temperature overnight and then neutralizing to pH 7·0. Bioassay showed that no detectable biological activity (i.e., less than 0·1 per cent penicillin) remained. Concentrations are expressed in terms of the penicillin solution from which it was derived, as units-equivalent/ml.

S fixation by cells was followed under conditions previously used for pre-treatment of cells for penicillinase adaptation experiments (Pollock, 1950)—viz., in M/100 buffer at 0°, usually for 1 hr., followed by 3 washings. The only variation from the previously standardized technique was that cells were treated at a concentration of 2 mg./ml. instead of 1 mg./ml., in order to economise in penicillin.

RESULTS.

Effect of penicillin concentration on S fixation.

Fig. 1 shows the amount of S fixed by the cells after treatment for 1 hr. at 0° with different concentrations of radioactive penicillin followed by 3 washings.

The general form of the curve is not dissimilar to that found for penicillin-sensitive *Staph. aureus* by Rowley *et al.* (1950). There is a similar disproportionately high uptake at low concentration of penicillin, and the total amount of S bound/mg. cells is of the same order. There is a fairly definite point of inflection in the curve at about 1 unit/ml., above which the amount of S fixed is directly proportional to the concentration of penicillin used for treatment. The shape of the curve suggests that there are two types of S-fixation processes occurring: (*a*) a reaction with some receptor which becomes saturated at about 1·0 unit/ml. and will therefore take up no more S from penicillin above that concentration, and (*b*)

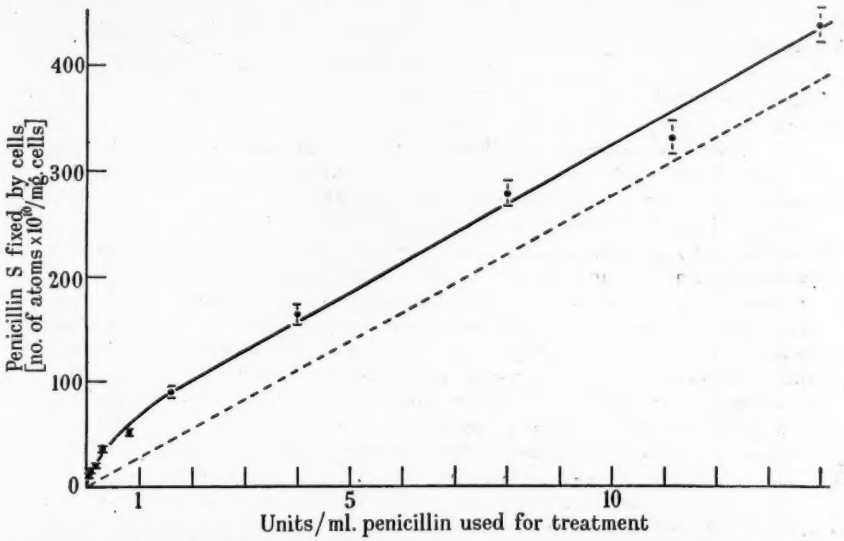

FIG. 1.—Fixation of S by *B. cereus* cells treated with S^{35}-labelled penicillin for 1 hr. at 0° followed by 3 cell washings. Limits of counting error are shown for P = 0·05. An interrupted line has been drawn through the origin parallel to the linear portion of the curve, to indicate the amount of " non-specific " S-fixation (see text).

fixation on to some other cell material which shows no sign—up to a level of 14 units/ml., at least—of being limited with respect to the amount absorbable.

That such an interpretation is reasonable is supported by results shown in Fig. 2 where the S-uptake curve from penicillin is compared with that obtained, under similar conditions, from equivalent concentrations of penicilloic acid. S-fixation from penicilloic acid is directly proportional to the concentration with which the cells are treated over the whole range tested, and is of the same order as the " non-specific " fraction of S-fixation from penicillin. The remaining S bound from penicillin is presumably specific for that compound and is of particular interest, since the fixation reaction appears to be complete at a level of about 1·0 unit/ml.—the concentration at which maximal effect on penicillinase production rate has already been shown to occur.

Errors inevitably associated with such fixation experiments do not allow a

very accurate estimation of the penicillin concentration at which maximal specific S-fixation occurs. But the curves illustrated in Fig. 1 and 2 have now been repeated 8 times with consistent results; and it is reasonable to put the probable level at between 0·5 and 2·0 units/ml.

Specific fixation of Penicillin S.

Fig. 1 and 2 show that $2{\cdot}5 \times 10^{11}$ atoms of S/mg./unit/ml. are fixed "non-specifically" from penicillin. If this quantity is subtracted from the curve in

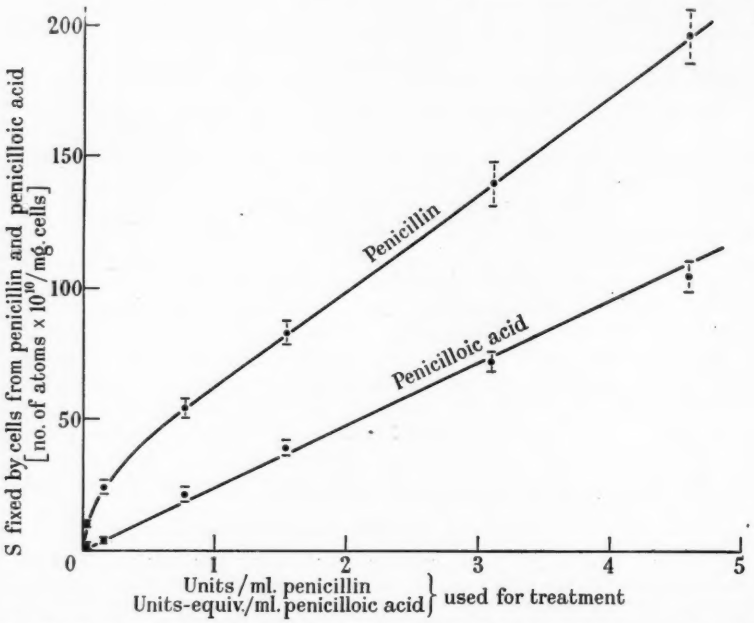

FIG. 2.—Fixation of S from S^{35}-labelled penicillin and penicilloic acid by *B. cereus* cells, showing limits of counting error for P = 0·05.

Fig. 1, it is possible to derive a curve for the specifically bound penicillin S—as shown in Fig. 3. In the same figure values are also plotted for penicillinase production rates obtained in the same experiment after treatment at 5 different penicillin concentrations (0, 0·08, 0·8, 8·0 and 14·0 units/ml.). It is, unfortunately, impossible to carry out the enzyme adaptation experiments at the same time at a greater number of different penicillin levels, and this figure must therefore be compared with the composite graph obtained from a number of different experiments published previously (Pollock, 1950). It does, however, clearly show maximal specific S-fixation occurring at a penicillin pre-treatment concentration of about 1·0 unit/ml.—the same value as for maximal stimulation of penicillinase production.

Rate of S-fixation from Penicillin.

Two lots of 30 ml. of cells at 2 mg./ml. were treated at 0° with 0·1 and 1·0 unit/ml. radioactive penicillin respectively and 10 ml. samples removed at 10, 60 and 180 minutes, spun down as rapidly as possible (6 minutes) and washed 3 times with water as usual. The amount of S fixed was then measured and the results plotted against time as shown in Fig. 4. At 1·0 unit/ml., after 10 minutes, the amount of S fixed was 48·5 per cent of that fixed after 3 hours, and after 60 minutes the percentage had risen to 73. It is clear that penicillin reacts with some cellular component very rapidly. The curve should be compared with that for penicillinase formation rates following identical penicillin treatment (Pollock, 1950) where the corresponding figures were 41 per cent and 67 per cent respectively. The correspondence between the two is striking.

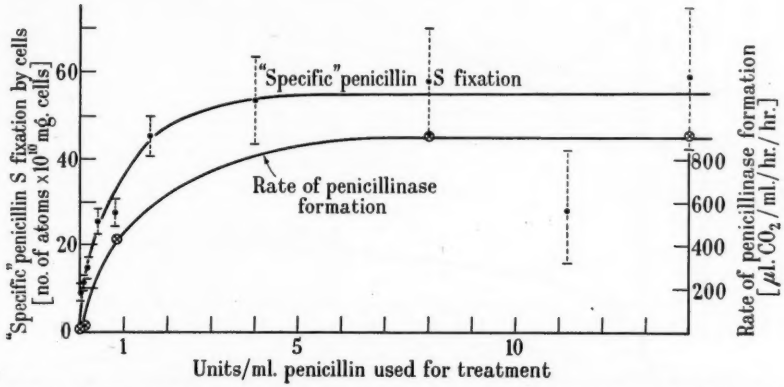

FIG. 3.—" Specific " fixation of S on to cells and rates of penicillinase formation following treatment of *B. cereus* with S^{35}-labelled penicillin. Limits of counting error are shown for P = 0·05.

Retention of fixed S during subsequent cell growth.

After standard treatment of cells with 0·5 unit/ml. S^{35}-labelled penicillin, they were inoculated at 1 mg./ml. into 0·8 per cent casein hydrolysate + M/50 glucose medium and incubated at 35° aerobically on a shaker. Samples were removed at 0, ½, 1, 1½, 2 and 2½ hours, the cells spun down, washed once and fixed radioactivity determined. The results are shown in Fig. 5 as total S fixed on cellular matter, derived (originally) from 1 mg. dry weight of cells, against time. Since during the 2½ hour period cell mass increased about 3-fold, the fixed S would be diluted in the growing cell protoplasm and results expressed per mg. of cells (as previously) would be confusing. The curve suggests that only about 30 per cent of S is probably lost during this period, and that therefore the greater part must be very firmly bound to cell material. Similar retention of penicillin S by *Staph. aureus* during subsequent growth in a penicillin-free medium has been reported by Maass and Johnson (1949*b*).

Proportion of penicillin S fixed on cells.

The penicillin concentration/S-fixation curve in Fig. 1 shows that about 2·5 $\times 10^{11}$ atoms of S per mg. of cells are fixed non-specifically from 1 unit/ml. and that the amount fixed specifically varies from 9×10^{10} atoms/mg. at 0·04 unit/ml. to about 50×10^{10} atoms/mg. at 1·5 units/ml. and above. The total penicillin S fixed on to cells treated at 2 mg./ml. varies from 0·50 per cent of that present in the medium at 0·04 unit/ml. to only 0·063 per cent at 14 units/ml. The proportion is thus exceedingly small. Assuming the average cell weight of *B. cereus* to be about 2×10^{-13} g., it appears that there are of the order of only 25 atoms of S specifically fixed per cell after treatment with 0·1 unit/ml. and 80 atoms/

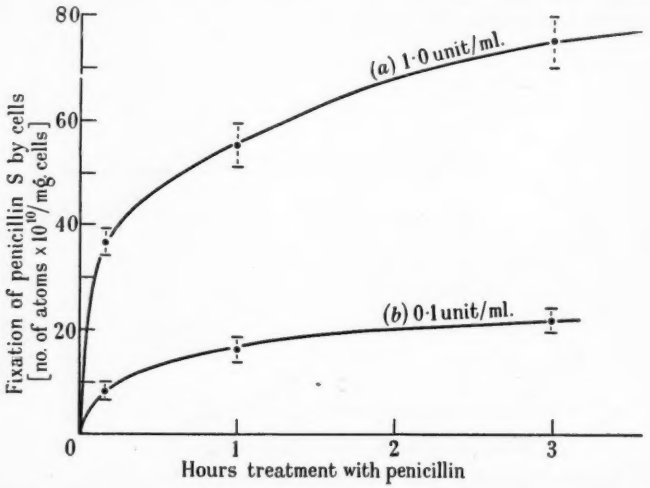

FIG. 4.—Rates of S fixation by *B. cereus* cells treated with S^{35}-labelled penicillin (*a*) at 1·0 unit/ml., (*b*) at 0·1 unit/ml. Limits of counting error are shown for P = 0·05. Penicillin assayed at 77 per cent nominal value.

cell after treatment with 1·0 unit/ml. (level at which maximal specific fixation and maximal penicillinase adaptation stimulation occurs).

Since measurement of S-fixation gives no information about the fate of the remaining penicillin S, attempts were made to estimate the actual amount of intact penicillin left in the medium after treatment of cells for 1 hour at 0° with 0·5 unit/ml. This level was chosen as being high enough to obtain reasonably accurate results by bio-assay, and yet still within the range known to be limiting both for specific S-fixation and penicillinase adaptation.

After treatment at 0·5 unit/ml. the cells were spun off as rapidly as possible in the cold room and the supernatant fluid divided into 2 parts. One part was heated for 30 minutes at 60° and then assayed on plates as described in " Methods." The preliminary heating was found necessary in order to avoid substantial loss of activity after cell treatment and during assay. This protective effect was probably due to destruction of traces of penicillinase already present, and to delay of

growth of the few living cells inevitably left behind in the supernatant after centrifugation. Both of these factors might be expected to contribute to the inactivation of penicillin during incubation of the assay plate. The other part of the supernatant fluid was used to treat a second lot of cells under the same conditions as the first. The two lots of cells thus treated for 1 hour at 0° [viz., (*a*) with penicillin at 0·5 unit/ml. and (*b*) with supernatant fluid from (*a*) after removal of the first lot of cells] were spun, washed 3 times, and their rates of penicillinase formation measured in CH at 35° as described in " Methods." The results are given in the Table. In two experiments there was a mean loss of 20

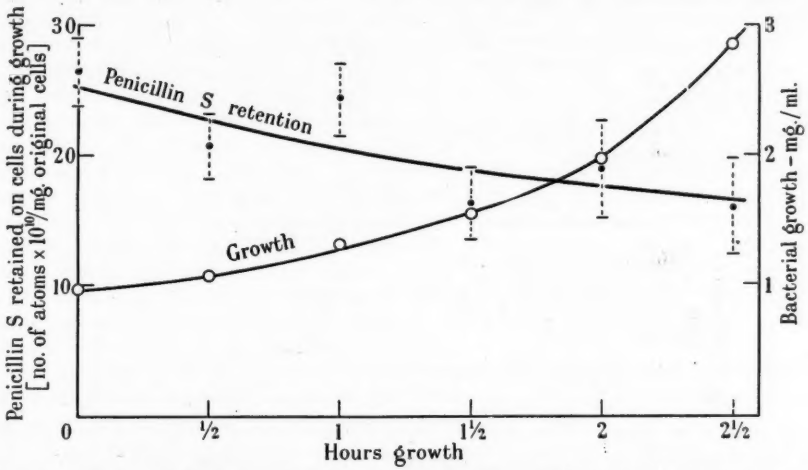

FIG. 5.—Loss of penicillin S from *B. cereus* cells during growth in casein hydrolysate following treatment for 1 hr. at 0° with 0·5 unit/ml. S^{35}-labelled penicillin. Limits of counting error are shown for P = 0·05.

per cent and 18 per cent respectively in antibacterial activity, and a falling off of only 9 per cent in ability to stimulate penicillinase adaptation (done in Expt. IV only). The limited accuracy of the technique is such that these results are consistent with the loss being, in both cases, little more than the fraction of penicillin S known to be actually fixed on the cells during pre-treatment.

DISCUSSION.

The general characters of S-fixation from penicillin by *B. cereus* are not dissimilar from those reported by Rowley *et al.* (1950) for other bacteria, although the total amount bound at very low penicillin concentration (0·04 unit/ml.) is 1/10 to 1/20 of what these workers found with penicillin-sensitive *Staph. aureus*. The shape of the curve of relationship between S fixed and concentration of penicillin used for treatment justifies its resolution into two components: (1) " specific " fixation with a maximum binding at about 1·0 unit/ml., and (2) " non-specific " fixation directly proportional to penicillin concentration over the

TABLE.—*Loss of Penicillin During Treatment of* B. cereus *with 0·5 unit/ml. for 1 hr. at 0°.*

Test used.			Cell concentration during treatment (mg./ml.).
A. Proportion of penicillin sulphur fixed on cells :			
Expt. I	0·16%	—	2·0
Expt. II	0·45%	—	5·0
B. Loss of antibacterial activity :			
	Mean loss from medium after treatment.	Limits of loss for P = 0·05.	
Expt. III	20%	10%–29%	1·0
Expt. IV.	18%	10%–25%	1·0
C. Loss of ability to stimulate penicillinase production :			
	Solution used for pre-treating cells.	Subsequent rate of enzyme formation in casein hydrolysate (μl. CO_2 ml./hr./hr.).	
	(*a*) Penicillin 0·5 unit/ml.	920	1·0
	(*b*) Supernatant from (*a*) after removal of treated cells	840	
	(*c*) Water (control)	10	
Loss of activity after treatment : 9%.			

whole range of values (from 0·04 to 14 units/ml.) tested. The fact that there is no corresponding "specific" S-fixation from penicilloic acid supports the conclusion that the "specifically-bound" fraction of penicillin S is due to an independent interaction between penicillin and some cell receptor specific for penicillin alone.

Comparing the two specific effects of penicillin treatment of *B. cereus*, (*a*) fixation of penicillin S by the cells and (*b*) stimulation of subsequent rate of penicillinase formation, it can be seen that—

(1) Both effects are maximal at a penicillin concentration of about 1·0 unit/ml. (Fig. 3).

(2) Both effects proceed at about the same rate (Fig. 4 and see Pollock, 1950).

(3) Both effects are more or less irreversible in the sense that bound S is retained (with little loss), and penicillinase continues to be formed at approximately constant rate, during subsequent growth in penicillin-free CH medium (Fig. 5 and see Pollock, 1950).

The significance of close correlation in (2) is somewhat less than might at first be expected, since it is possible that both penicillin effects might, initially, be limited by the rate of penetration of the penicillin into the cell. If this is so, all that is being measured in (2) is the speed of permeation of penicillin through the cell wall and membrane, and the only justifiable conclusion is that both effects take place very rapidly.

Considering the possible range of differences between the two effects in general the correlation is striking, and strongly suggests that they are not independent phenomena. Attempts, however, to show that all variations in the adaptive response of cells to penicillin treatment were due merely to differences in the amount of S specifically fixed were not successful. Different batches of cells and identical batches treated in different ways (e.g., with 1 hr. pre-incubation in CH medium + M/50 glucose) may differ considerably (5-fold or more) in their absolute rates of penicillinase formation following treatment with the same penicillin concentration, although they all show maximum response at about 1·0 unit/ml. Such variations were not associated with proportional variations in the quantity of specifically bound S. The latter, in fact, appeared to be fairly constant from one experiment to another, maximum variations not exceeding 30 per cent.

Nevertheless, the conditions affecting specific S-fixation are very similar to those pre-determining rate of penicillinase formation. The parellelism is too close to be merely coincidental, and indicates that the substance with which the penicillin reacts, when it exerts its effect, is qualitatively identical in the two instances. It seems reasonable, therefore, to conclude that the interaction between penicillin and some specific cell receptor which results in fixation of penicillin S is a necessary preliminary to the specific stimulating effect on subsequent penicillinase formation in a penicillin-free medium. The fact that there is not always a close quantitative relationship between the two effects only shows, as might be expected, that there are additional factors influencing penicillinase adaptation about which little is yet known. Moreover, the hypothetical S-fixing receptor with which the penicillin reacts exists in many species of bacteria—e.g., penicillin-sensitive *Staph. aureus* (Rowley *et al.*, (1950)—which do not produce any penicillinase whatever, adaptive or otherwise. It cannot therefore be the sole hereditary factor determining penicillinase adaptability.

It is not perhaps too early to speculate on whether some similar interaction between substrate and cell receptor might be the first essential preliminary to other enzyme adaptations in micro-organisms. If so, it would be neccesary to assume, on existing evidence, that the reaction is reversible, and in that case would be difficult to demonstrate. For instance, *Bact. coli* " 1433," if treated with nitrate, is not thereby endowed with any increased ability subsequently to form the adaptive nitratase in a nitrate-free medium (Wainwright and Pollock, 1949). The case of penicillinase may be a special one, and it is essential to make the qualification that the reaction between penicillin and receptor, which results in specific S-fixation, may not be necessary for penicillinase formation in the presence of excess free penicillin, though this possibility appears unlikely. Nothing is yet known about the extent to which the penicillin molecule is modified by its reaction with the cell. It is thus possible that the function of the specific receptor in penicillinase adaptation is simply protective—by retaining a small quantity of penicillin in a state in which it can constantly exert its catalyst-like activity in stimulating penicillinase formation after the removal of free penicillin. What is of particular interest, however, is the clear evidence that the ability of *B. cereus* cells to produce greatly increased amounts of an enzyme under certain defined conditions depends in the first place upon a definite chemical or physicochemical reaction between the specific substrate and some substance already present within the cell. This focuses attention upon the nature and significance of the receptor substance, and the type of reaction it undergoes with penicillin.

Further information on this reaction should greatly assist in the study of the other factors which appear also to be necessary before the cell is able to produce penicillinase at maximal rate on subsequent incubation.

SUMMARY.

1. The uptake of S^{35} by *B. cereus* cells has been measured after treatment at 0° with varying concentrations of S^{35}-labelled benzyl penicillin and benzylpenicilloic acid and subsequent washing.

2. The proportion of S fixed from penicillin is very small, and decreases as the penicillin concentration increases, varying from 0·50 per cent of the total at 0·04 unit/ml. to 0·063 per cent at 14 units/ml., using a bacterial suspension of 2 mg./ml.

3. S-fixation from penicillin is made up of two superimposed fractions: (*a*) non-specific fixation—directly proportional to penicillin concentration in the medium and of the same order as the total S-fixation from penicilloic acid; and (*b*) specific fixation with maximum absorption at 1·0 unit/ml.

4. There is a correlation between the specific fixation of S by cells from penicillin and the effect of penicillin in specifically stimulating the subsequent rate of penicillinase formation, in that both processes (*a*) have a maximum at a penicillin concentration of 1·0 unit/ml., (*b*) proceed at the same rate, and (*c*) are more or less irreversible during subsequent incubation and cell growth for 2½ hours in casein hydrolysate.

5. It is concluded that the process entailing specific fixation of S by the cells from penicillin is essential to its action as a specific stimulator of penicillinase production in the absence of free penicillin.

We are deeply indebted to Dr. E. Lester Smith, of Glaxo Laboratories Ltd., for generous gifts of crystalline S^{35}-labelled penicillin. We should also like to thank Dr. G. Popjak for invaluable advice on radioactivity measurements, Dr. W. L. M. Perry for statistical analysis of the bioassay results, Dr. J. H. Humphrey for advice on the technique of penicillin assay, and Miss Daphne Towler for technical assistance.

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ON THE ASSESSMENT OF THE INFLUENCE OF CHEMICAL COMPOUNDS ON BACTERIOPHAGE MULTIPLICATION.

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THE influence of chemical compounds on the multiplication of bacterial viruses (bacteriophages) can be studied by using phage strains which have a well-defined lytic cycle of development.

In the free state phage particles remain viable for a long time without multiplying or, as far as can be ascertained, altering in any way. When a free particle comes into contact with a sensitive host-bacterium it is adsorbed and subsequently penetrates the cell membrane. Knowledge of the exact behaviour of the phage from this point until the liberation of a crop of similar particles when the bacterium ruptures is still incomplete. One positive observation is that during an interval which probably occupies the greater part of this stage it is impossible to recover active phage particles from the bacterium (Doermann, 1947–48). There is no convincing evidence that multiplication takes place by binary fission. It has been postulated that the phage loses its identity and assumes the role of a dominant gene (Luria, 1950). Under its influence, enzyme systems of the bacterium are deflected from their normal functions and build up, partly at least from materials present in the culture medium (Kozloff and Putnam, 1950; Kozloff, Knowlton, Putnam and Evans, 1951), the different chemical components which go to make up phage. These include desoxyribosenucleic acid, a substance which is not normally found in any quantity in the bacterium, but which is one of the main constituents of phage. When these components have been accumulated in adequate amounts they become organized into particles. Finally the bacterial cell-membrane ruptures, and mature phage particles are liberated, thus completing the cycle of development.

Phage can be attacked at three points in this cycle: it may be destroyed in its free state; adsorption to the bacterium may be blocked; or the synthesis of new phage substance within the bacterium may be prevented. The first two may be dismissed briefly, as the work to be described relates to the third.

Phage in its free state has considerable powers of resistance to direct chemical action. Kreuger and Elberg (1934) showed that coliphage suspension was inactivated by mercuric chloride and potassium cyanide, and this inactivation was reversible upon precipitation of the Hg^{++} and CN^{-} groups respectively. Goldsmith (1935) found that a staphylococcal phage was destroyed by phenyl mercuric chloride. Kendall and Colwell (1938) showed that certain aldehydes inactivated phage. Some dyes have a direct action on phage (Kreuger and Baldwin, 1935; Walker, 1936; Wells and Sherwood, 1934). In the present investigations it has been found that phage is destroyed by certain of the phenanthridinium compounds in a concentration which allows of bacterial multiplication.

In most of the instances which have been quoted the concentration of the chemical necessary for phage destruction has to be of an order which is toxic to living cells, and consequently the inhibition or destruction of phage in this way is of limited interest.

Adsorption of certain phages can be prevented in artificial culture by withholding substances such as tryptophane, which must be present in small quantities before this process can take place (Anderson, 1945). This method of controlling phage multiplication has not been widely explored.

Before discussing the third method—interference with phage synthesis within the infected organism—certain basic principles must be considered. As phage multiplication is dependent on the enzyme systems of the host bacterium, it follows that any substance which grossly interferes with the bacterial metabolism may have a corresponding but indirect action on the phage. It is misleading to regard an effect which arises from damage to the host bacterium as a straightforward case of phage inhibition. A few compounds are, however, capable of producing well-marked changes in the degree of phage production without seriously affecting the growth of the organism. Such action is usually confined within certain limits of concentration, above which there is inhibition of both organism and phage and below which there is inhibition of neither. Any technique designed to test the phage-inhibiting properties of a compound must therefore embody controls to show its action on the normal host bacterium when the latter is growing in conditions identical with those of the test.

On reviewing the literature it is found that this limiting factor has frequently been overlooked. Many experiments have been designed in such a way as to give no reliable indication of the effect of the drug on the normal bacterium.

Nicolle and Mimica (1947) attempted to show the inhibitory action of certain antiseptics (formalin, perchloride of mercury, phenol and gonacrine) by placing drops of different concentrations on a plate sown with phage-infected bacteria. The underlying principle is that, on incubation, those portions of the plate which are unaffected by the drug remain clear because the bacteria are destroyed by the phage and so fail to grow ; if the drug has both antiphage and antibacterial action the latter, of itself, prevents bacterial growth, and thus obscures the antiphage action which, in the circumstances, is of no interest ; but if the chemical has antiphage action without being antibacterial, the area under its infleunce shows normal bacterial growth. In the experiments carried out by Nicolle and Mimica, a ring of colonies formed at some distance from the centre of a drop of certain concentrations of the antiseptics used. They concluded that this area represented the point at which the concentration of the drug had been sufficiently reduced by diffusion through the agar to inhibit phage only. This test is qualitative in nature rather than quantitative. It gives no more than a rough indication of the concentration at which the drug inhibits phage action without being wholly antibacterial, and no reliable indication of partial antibacterial action. A similar technique was used by Asheshov, Strelitz and Hall (1949) in searching for antibiotics active against phage. In their work the test was used qualitatively and not quantitatively.

After preliminary trials by Fitzgerald and Babbit (1946) a series of experiments was carried out by Fitzgerald and Lee (1946) using *Bact. coli* and a phage to which it is sensitive. The organism and phage were cultivated in a synthetic medium, and a series of acridines was tested at concentrations which had been

shown in the preliminary experiment to be the maximum which did not significantly affect the growth of the bacterium alone. By this method it was believed that true phage inhibition was demonstrated. The antiviral effect of the active acridines was found to be counteracted by ribosenucleic acid, and the authors conclude that this indicates interference with some mechanism concerned in nucleic acid synthesis. Foster (1948) investigated the action of proflavine sulphate on the T series of coliphages, using the one-step growth technique of Delbruck and Luria (1942). She found that growth, particularly of the " large-particle " types (T2, T4, T6), is completely inhibited by drug concentrations that allow normal and subnormal multiplication of the host bacterium, and also that the drug appears to block some late reaction necessary for the production of active phage. It is suggested that the blocked reactions are associated with the formation or utilization of desoxyribosenucleic acid. Cohen and Anderson (1946) found that 5-methyl tryptophane inhibited the multiplication of *Bact. coli* B, and that organisms in the presence of this compound were unable to produce T2 and T4 phage when infected therewith. The reaction is reversible by the addition of tryptophane to the medium. This is primarily an antibacterial action. Dickinson (1948) tested a large number of compounds on a system of *Ps. pyocyanea* and a specific phage. Agar from which plates were prepared was impregnated with serial concentrations of the drug under test, and sown either with lysogenic strains showing visible signs of phage activity or with bacteria infected with the phage. Absence of silvery sheen on the lysogenic cultures and absence of lysis in the others were taken as evidence of activity. The test is not delicate and is only partly quantitative. The information regarding the development of the bacterium is inadequate and the test does not show minor degrees of deviation from normal. Czekalowski and Dolby (1949) studied the action of various enzyme inhibitors on the development of T2 phage in *Bact. coli* B. A phage-infected culture was incubated for $2\frac{1}{2}$ hours, then divided up and each portion subjected to the action of the various inhibitors for 1 hour. The effect of the drug was then estimated as regards action both on bacterium and on phage. Details of the method of controlling the action of the drug on bacterial growth are not given, but certain of the concentrations mentioned appear unduly high.

Most of these methods are lacking in precision, and either do not give due consideration to the antibacterial as opposed to the antiphage action of the compounds, or else fail to include controls capable of showing which of these types of action is responsible for the results. Nevertheless, they do appear to show that certain compounds are capable of modifying or inhibiting phage multiplication at concentrations which have no obvious harmful action on the bacterial metabolism. It seemed of interest to devise more precise methods for studying this action, and the work now to be recorded was undertaken with this object.

MATERIALS.

Culture media and technique.

The culture media and methods used were, in general, those described in the literature, with minor modifications which have been given elsewhere (Boyd, 1950). Estimations of turbidity were made with the Spekker absorptiometer.

Bacteriophage system.

For most of this work we have used *Salm. typhi murium* and a phage (No. 196) which is commonly found in close association with this organism in infected mice. When suitably balanced quantities of bacteria and phage are introduced into broth, a period of growth and increasing turbidity is followed by clearing which to the naked eye is practically complete. The following standard technique was used throughout. *S. typhi murium* (Strain 1404) was grown overnight on an agar slant, then washed off with normal saline, and the number of organisms per ml. was estimated by means of the absorptiometer. The suspension was diluted so that 0·5 ml. contained approximately 5×10^8 bacteria. This quantity was pipetted into 4·5 ml. of broth, giving a concentration of 1×10^8 organisms per ml. To this was added a standard loop of phage diluted so as to give a final ratio in the culture of approximately one phage particle to 10,000 organisms. When such a culture is incubated, turbidity increases for from 2 to 3 hours, and thereafter a gradual lysis occurs. To avoid the confusion caused by secondary growth, the tubes were given 7 hours' incubation and placed in the refrigerator overnight, during which time lysis reached its limit and the opacity thereafter remained stable at this temperature.

Slow lysis such as this is considered to be of advantage because it gives the compounds which are being tested every opportunity to exercise any inhibitory action they may possess.

Bacterium-bacteriophage systems of *Bact. coli* B with phage T2 and *Shigella dysenteriae* with an active Shiga-phage have also been used. The former is unsatisfactory because clearing is abrupt and minor degrees of inhibition are not well recorded. The latter is often of little use because the bacterium is unduly susceptible to the action of the compounds which are being tested. It is justifiable to assume that, as the metabolism of each organism, and possibly of each type of phage, is different, so the details of the findings can be applicable only to the particular bacterium-bacteriophage system used. Even so, it may be anticipated that different systems will show many points of similarity.

METHODS.

After a number of preliminary trials a method (Method 1) was devised which made it possible to differentiate between the antibacterial and the antiphage action of any compound. The technique of this method has the disadvantage of being somewhat laborious and the test has occasionally had to be carried out in two stages, a preliminary run with widely spaced concentrations of the drug and a second with concentrations more closely spaced round the apparent optimum. From observations made when carrying out a large number of these tests an alternative and less time-consuming method (Method 2) was devised by one of us (P. L. B.) which gives satisfactory results with this particular phage system.

Method 1.—This is a straightforward comparison of the growth curves (turbidity) of a normal culture and a phage-infected culture in medium containing different concentrations of the drug. In the screening test two sets of four tubes of broth medium are used, No. 1 tube being a normal control and Nos. 2, 3 and 4 containing drug concentrations of 1/10,000, 1/100,000 and 1/1,000,000 respectively. The first set of tubes is inoculated with bacteria alone, the second with bacteria

and phage as already described. Readings are made before incubation and thereafter at hourly intervals for 7 hours when the tubes are placed in the refrigerator overnight. Next morning (24 hours from the beginning of the test) a final reading is taken. Curves are then plotted, one series for the control tubes and the other for the test (Fig. 4, 5, 7, 9). The phage content of the second series of tubes is estimated first by rough titration and then, if necessary, by counting the phage particles per ml. In this test inhibition of phage action and its relationship to the antibacterial action of the compound can be assessed by comparison of the two sets of curves, while interference with phage multiplication is shown by the final titrations.

In certain cases it will be apparent that the optimum concentration of drug for phage inhibition lies between two of those used in the screening test, and further tests with intermediate quantities must be carried out.

Method 2 is based on the degree of turbidity reached by a bacterium-bacteriophage system in the presence of different concentrations of the drug when a standard method of incubation (as in Method 1) is used. The underlying principles of the test are as follows: If a chosen organism is cultivated in different concentrations of an inhibitory drug, growth curves as shown in Fig. 1(*a*) will be obtained. Each lies above the other in definite order, the highest concentrations of drug being associated with the most restricted growth, and *vice-versa*. Under standard conditions the end-point of each curve is relatively constant, and by using six tubes for each drug concentration and striking the mean of the readings, a high degree of consistency is achieved. These end-points may now be arranged on a new graph (Fig. 1(*b*)) in which they are the ordinates, while the drug concentrations become the abscissae.

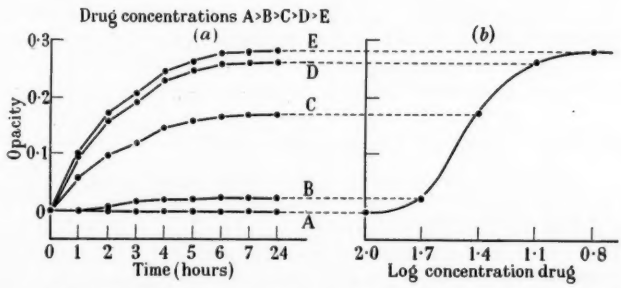

FIG. 1 (schematic).—(*a*) Turbidity curves indicating the degrees of bacterial growth in cultures containing different concentrations of drug. (*b*) The end-points of these curves plotted against the concentrations of the drug.

If to a second identical series of cultures a measured quantity of phage is added before incubation, an equally constant series of curves and end-points of turbidity will be obtained. The curves and end-points can be similarly plotted (Fig. 2(*a*) and (*b*)). The result, when an active drug is used, is a somewhat cone-shaped curve, the rising or left-hand limb of which represents increasing bacterial growth in decreasing drug concentrations which are nevertheless sufficient to cause complete suppression of phage action, while the falling or right-hand

limb shows the increasing phage action which takes place as the drug concentration falls below the point at which it causes phage inhibition.

From the combined curves of the series without phage and the series with phage (Fig. 3) considerable information can be gleaned. The point at which the curves leave the base line and start to rise shows the toxicity of the drug for the bacterium—the higher the drug concentration at this point the lower the toxicity. The point at which the two curves part company corresponds with the concentration of the drug which just inhibits phage action. The horizontal distance between these two points shows the range of concentrations at which the drug

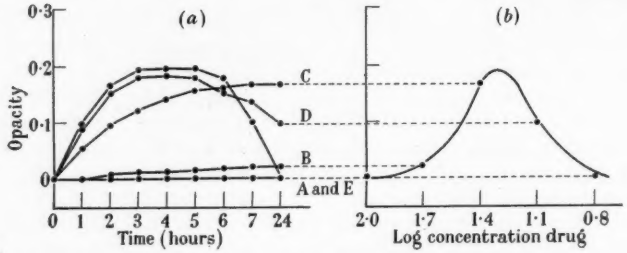

FIG. 2 (schematic).—Similar to Fig. 1, but with added bacteriophage.

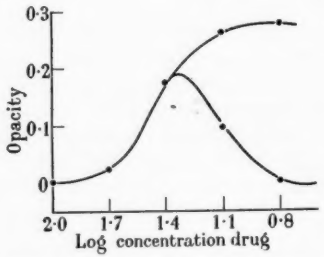

FIG. 3 (schematic).—Figs. 1(*b*) and 2(*b*) superimposed.

inhibits the phage while still permitting bacterial growth. The height of the second point in relation to the final constant height of the bacterial growth curve gives the percentage of bacterial growth which occurs before lysis begins. When the horizontal distance is short and the peak of the curve is low the drug which is being tested has little antiphage action. Indeed, any which it appears to possess may in reality be due to direct action on the bacterium. On the other hand, when these points are well separated and the peak is high, reaching to the upper limits of the control curve, it may be concluded that a true antiphage action is present, as normal bacterial growth is taking place in drug concentrations which are sufficient to prevent multiplication and bacterial lysis.

Both methods must be controlled by tests to exclude direct action on free phage and interference with adsorption. If these are negative, any activity which is found must be attributable to interference with the process of multiplication within the bacterial host.

EXPERIMENTAL FINDINGS.

These methods have been used to examine a range of compounds, some of which have been claimed by other workers to show antiphage action, while others have not previously been investigated. Different degrees of action have been found which, for convenience, may be classified into groups.

(1) *Compounds showing insignificant antibacterial action and no phage inhibition.*

Colchicine is a good example of this group and the curves given by Method 1 are shown in Fig. 4. It has not been possible to examine this compound by

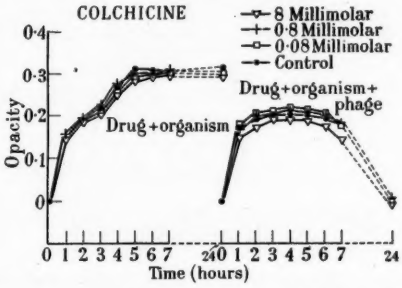

FIG. 4.—Turbidity curves showing that colchicine is without action on this phage.

Method 2, as the antibacterial action at high drug concentrations is too trivial to permit of a curve being plotted, and antiphage action is absent. Colchicine was selected as it was claimed by Czekalowski and Dolby (1949) to produce inhibition of coliphage T^2 in 0·0004 millimolar concentration. No such activity is shown against phage 196 in solutions containing many times this concentration.

(2) *Compounds showing varying degrees of antibacterial action but no phage inhibition.*

Pentamidine (Fig. 5 and 6) shows increasing degrees of antibacterial action without any appreciable antiphage action. Thus a concentration of 0·01 per

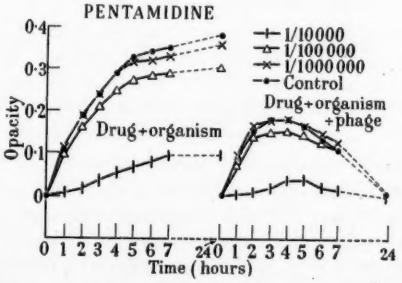

FIG. 5.—Turbidity curves showing that pentamidine is antibacterial without having antiphage action.

cent produces a marked degree of bacteriostasis, yet lysis occurs and advances to the same point as in the control. Further evidence of lack of antiphage activity, not shown in the figure, is afforded by the final bacteriophage count; this is alike in all cultures, irrespective of drug concentration. The flat course

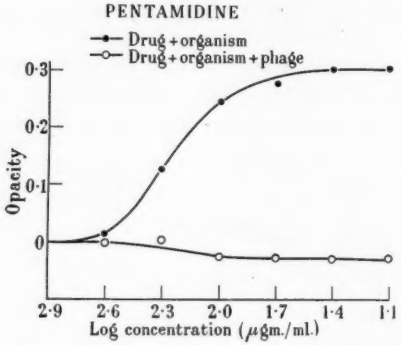

FIG. 6.—Pentamidine investigated by Method 2. Well marked antibacterial action but no antiphage action.

of the drug + organism + phage curve in Fig. 6 is characteristic of drugs showing no antiphage action.

(3) *Compounds showing antibacterial action and varying degrees of phage inhibition.*

This type of reaction can be seen in the curves given by the antibiotic terramycin, which has a powerful antibacterial action (Fig. 7 and 8). No phage

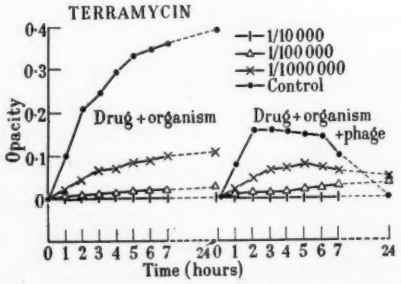

FIG. 7.—Turbidity curves showing powerful antibacterial action and limited antiphage action of terramycin.

action takes place in concentrations of the drug which are completely or almost completely bacteriostatic, but where moderate bacterial growth is present phage multiplication and lysis occur. In the experiment illustrated in Fig. 7 the phage particle counts per ml. after incubation were *nil* in concentrations of 1/10,000 and 1/100,000 per cent. and 1×10^7 in 1/1,000,000, while the control reached 2×10^{10}. The low peak given by Method 2 is characteristic. It seems probable that this degree of phage inhibition is attributable to direct action on the

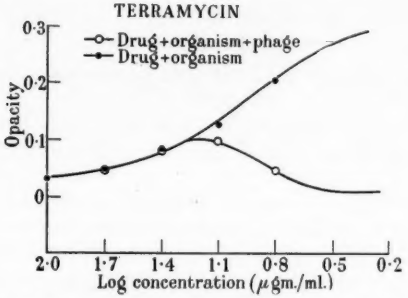

FIG. 8.—Terramycin investigated by Method 2.

metabolism of the bacterium with a secondary effect on the phage rather than to some process directed specifically against the phage itself.

(4) *Compounds having little or no antibacterial action in concentrations which completely inhibit phage.*

Rivanol (2 : 5-diamino-7-ethoxyacridine lactate) is the best example of this group which has been encountered (Fig. 9 and 10). At a concentration of 1/20,000

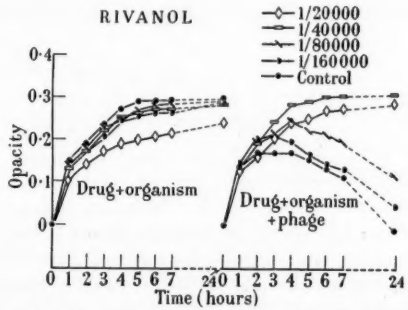

FIG. 9.—Turbidity curves showing that Rivanol has well-marked antiphage action at concentrations which have little effect on bacterial growth.

it causes slight inhibition of bacterial growth and complete inhibition of phage multiplication, while at 1/40,000 the bacterial growth curve is little different from that of the control, and phage inhibition is still complete. At 1/80,000 and 1/160,000 this effect progressively disappears and the curves approximate to those of the control. The phage counts per ml. of the different concentrations are : 1/20,000 and 1/40,000, *nil* (no particles in a loopful) : 1/80,000, $2{\cdot}2 \times 10^{10}$: 1/160,000, 3×10^{10} : control $5{\cdot}4 \times 10^{10}$. Free phage particles are slightly affected after 24 hours' incubation with Rivanol in a strength of 1/1000, but not at lower concentrations.

When tested by Method 2 the two curves overlap to a point practically equal to the final density of the control, giving a high peak and a wide spread to the curve of the phage-infected series.

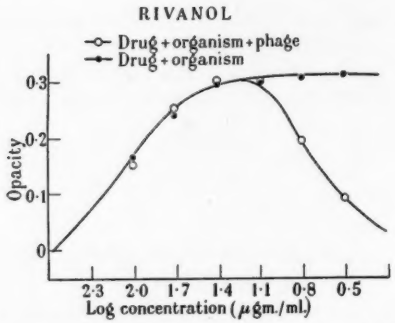

FIG. 10.—Rivanol investigated by Method 2. The high peak shows that Rivanol has antiphage action at a concentration which allows full bacterial growth.

A considerable range of compounds has been examined by these methods. Only a few have shown activity, and several, claimed by other workers to modify the multiplication of other types of phage, have had no apparent effect on the development of phage 196 in *Salm. typhi murium*. The action of sodium cyanide, for example, appears to be principally, if not solely, antibacterial, while that of proflavine is in part antibacterial and in part possibly a direct action on the free phage, although this is difficult to assess with certainty because of the antibacterial action of the drug.

DISCUSSION.

The object of the work described has been to devise and explore reliable methods for estimating the inhibitory action of chemical compounds on the multiplication of lytic bacteriophage, and it is not proposed to give lists of drugs which have been found to be active or inactive or to speculate on their mode of action. Both methods described are satisfactory. If carried out with the necessary controls they enable a distinction to be drawn between action which is directed against both bacterium and phage, and action which is specifically antiphage and has no important influence on the growth of the bacterium. Each method has its merits. When the approximate peak is known the second is probably more convenient, as half a dozen or more compounds can be passed through in a day without difficulty and with a minimum expenditure of time and labour. When an active substance has been detected by this method, its antiphage properties can be more accurately appraised by the first method.

A striking feature of the investigation has been the relatively large numbers of compounds which markedly slowed and in some instances all but abolished bacterial growth without producing a proportionate reduction in the final concentration of phage in the culture. It would appear from this that the enzyme systems concerned in promoting the growth of the bacterium are different from those misappropriated by the virus to further its own multiplication.

It must be emphasized that these results refer to one bacterium-bacteriophage system and do not necessarily apply to others.

SUMMARY.

1. Previous investigations into the action of drugs in inhibiting phage multiplication are reviewed.

2. Two methods of testing for antiphage action are described.

3. The results obtained by examining a wide series of compounds have been classified in four groups.

4. In a number of cases a marked degree of antibacterial action has little influence on phage production. In others depression of bacterial growth and of phage multiplication are approximately parallel. Very few drugs are capable of suppressing phage multiplication without also producing a reduction in bacterial growth.

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OVOMUCIN: A SUBSTRATE FOR THE ENZYME OF THE INFLUENZA VIRUS. III. ENZYMIC ACTIVITY AS AN INTEGRAL FUNCTION OF THE INFLUENZA VIRUS PARTICLE.

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FROM information obtained by the use of biological techniques in the study of different strains of influenza virus the concept has emerged that the influenza virus particle is endowed with enzymic function (for review see Burnet, 1951). Approaching the problem from the biochemical side Gottschalk and Lind (1949*b*) could substantiate the specific activity of an enzyme in virus preparations by isolating a dialysable nitrogen-containing carbohydrate released from certain semi-purified mucoproteins as a result of virus action. In recent experiments an electrophoretically homogeneous mucoprotein prepared from urine (Gottschalk and Ada, unpublished) by a slight modification of Tamm and Horsfall's (1950) procedure was used as substrate. The dialysable product liberated from the urine mucoprotein by a highly purified preparation of influenza virus was identified as an N-substituted isoglucosamine (Gottschalk, 1951). It would thus appear that no doubt is left with regard to the presence of an enzyme in the virus preparations used. There remained, however, the possibility, though remote, that the enzymic activity ascribed to the influenza virus might be due, not to the infectious particle itself, but rather to a separate entity contaminating the virus. In order to test this alternative interpretation of the experimental results the effect of purification of influenza A virus on the enzymic activity and the intrinsic virus property of haemagglutination was investigated, and the data thus obtained submitted to statistical analysis. Three series of experiments were carried out, an independent procedure of purification being applied in each of them. A fourth series of experiments was devised to test for the presence in normal host cells of an enzyme acting upon a suitable inhibitor of virus haemagglutination.

MATERIALS.

Preparation of ovomucin.

Ovomucin was prepared as described previously (Gottschalk and Lind, 1949*a*).

Growth of influenza virus.

The virus used in the experiments was the Melbourne strain of influenza A virus. Eleven or twelve days embryonated hens' eggs were inoculated with the virus and incubated for two days at 35°, after which period the allantoic fluid was harvested. This liquid is referred to as infected allantoic fluid (I.A.F.).

Purification of influenza virus.

Three different procedures were applied to purify the virus present in the infected allantoic fluid :

(1) In the first series of experiments virus was purified by specific adsorption on and spontaneous elution from erythrocytes. In this series an attempt was made to eliminate those virus particles with less than normal capacity to elute from red cells by an initial adsorption and elution into the parent allantoic fluid. In this way virus particles failing to elute spontaneously were removed from the preparation. A second cycle of adsorption and elution was then carried out in which saline (0·85 per cent NaCl solution) served as eluent. As the effect of the initial treatment seemed to be insignificant, it was omitted in the two other series.

(2) In the second series the virus was purified by adsorption on freshly precipitated calcium phosphate, the precipitate then being dissolved in ammonium citrate solution, as described previously (Gottschalk and Lind, 1949*a*).

(3) In the third series virus was purified by high-speed centrifugation, preceded by one cycle of adsorption on and elution from erythrocytes, the eluent being saline. Centrifugation was performed in an Ecco high-speed centrifuge with a centrifugal force of approximately 20,000 g. for 90 minutes at room temperature. The deposit containing the virus was suspended in saline and any insoluble material removed by low-speed centrifugation.

The technique used for adsorption and elution as carried out in (1) and (3) was the following (see Francis and Salk, 1942): To chilled I.A.F. were added packed fowl red cells to give a final concentration of 1 per cent per haemagglutinin titre of 190. After 30 minutes at 4° the virus-coated red cells were spun down in the cold, washed once with ice-cold saline and re-suspended in the appropriate medium (see (1) and (3)). The adsorbed virus was allowed to elute for two hours at 37°, when the virus eluate was separated from the cells by centrifugation. These conditions for adsorption and elution were found to give maximum adsorption (about 90 per cent) of the virus and a satisfactory yield in the eluate accompanied by a minimal degree of haemolysis.

METHODS.

Nitrogen determinations (in triplicate) were made as described previously (Gottschalk and Lind, 1949*a*).

Titration of virus haemagglutinin.

Virus haemagglutinin was titrated as done previously (Gottschalk and Lind, 1949*a*). The reciprocal of the dilution showing the end-point is referred to as the haemagglutinin (HA) titre. Each HA titre in the tables is the average of two titrations.

Estimation of the enzymic activity of the virus.

The enzymic activity of the virus was determined with ovomucin as substrate. Serial twofold dilutions of the virus were prepared in acetate-buffered saline (pH = 6·2) containing calcium, 5 to 10 inhibitory doses (I.D.) of ovomucin (see below) added to each dilution, and the mixture incubated at 37° for 1 hour in accordance with the technique of Stone (1949). The haemagglutinin inhibitory capacity of ovomucin remaining after virus action was titrated as previously (Gottschalk and Lind, 1949*a*). The Melbourne strain of influenza A virus was used

as indicator virus, prepared by heating I.A.F. for 30 minutes at 56° in the presence of citrate (final pH = 8·5) as described by Stone (1949). The amount of ovomucin (5–10 I.D.) required for the enzymic test was obtained by appropriate dilution of the original ovomucin previously titrated for its inhibitory capacity as above. The reciprocal of the virus dilution showing the end-point is referred to as enzymic activity (Enz.). Each result shown in the tables is the average of two estimations.

EXPERIMENTAL.

In the experiments summarized in Table I the virus was purified by specific adsorption on and elution from erythrocytes (see " Materials ").

The results recorded in Table II were obtained from experiments in which the virus was purified by adsorption on freshly precipitated calcium phosphate (see " Materials ").

In the third series of experiments the results of which are shown in Table III, the virus was purified by adsorption on and elution from red blood cells followed by a cycle of high-speed and low-speed centrifugation (see " Materials.")

The statistical evaluation of Tables I, II and III is given in Table IV.

In order to test the effect of breakdown of the cell structure on the virus haemagglutination inhibitor present as normal constituent inside the host cell the following experiment was carried out :

The chorio-allantoic membranes were removed from six embryonated hens' eggs, previously incubated for 13 days, and washed in isotonic saline. Three membranes were kept as controls, and the other three thoroughly ground with silica. Both groups were suspended in saline and incubated in the presence of penicillin for 18 hours at 37°. The control membranes were then ground with silica at 0° and re-suspended in saline. Finally the supernatants from the control and the test group were titrated for inhibitor of virus haemagglutination using the Melbourne strain as indicator virus. No significant difference was found, a representative pair of inhibitory titres being 1920 and 1360 respectively.

When at the end of the experiment the Melbourne strain of influenza A virus was added to the test group, the inhibitory titre was reduced to <10 after an incubation period of 4 hours (cf. Edney and Isaacs, 1950).

DISCUSSION.

It would appear from the results recorded in Tables I–III and evaluated in Table IV that three independent procedures of virus purification removing up to 99 per cent of the nitrogen of the crude virus preparation did not significantly alter the log of the ratio haemagglutinin titre/enzymic activity,—i.e., the relationship between an intrinsic quality of the virus particle and the enzymic activity displayed. The purification techniques applied included such discriminating processes as specific adsorption on and spontaneous elution from erythrocytes and high speed centrifugation. The results obtained make it highly improbable that the enzyme present in the influenza virus preparations is an entity apart from and existing side by side with the virus particle. That an enzyme protein molecule, minute in size as compared with that of an influenza virus particle (hydrated dimension = 123 mμ. diameter), should survive the selective purification methods used to approximately the same extent as the virus is hardly conceivable.

The question remaining is whether the enzyme associated with the influenza virus is an integral part of the virus particle, or whether it represents a component of the normal host cell which in the process of virus propagation becomes so firmly adsorbed to its surface as to follow the particle in unchanged proportion through the various purification steps. The same question has previously arisen with vaccinia virus. Thus purified elementary bodies of vaccinia were found to possess phosphatase, catalase and lipase (esterase) activity and to adsorb readily these enzymes from dilute solutions of them (Hoagland, Ward, Smadel and Rivers, 1942). Since the same enzymes abound in the host cells of the vaccinia virus the findings were interpreted to mean that the enzymes

TABLE I.*—*Correlation of Enzymic Activity with Haemagglutinin Titre. Effect of Purification by Adsorption of the Virus on and Elution from Red Cells.*

No.	Enz. titre (*a*).	HA titre (*b*).	b − a.	mg. N/ml.	Purification as p.c. N removed.†
1. Inf. allant. fl.	2·90	2·68	−0·22	0·599	
1st eluate	2·77	2·64	−0·13	0·600	
2nd eluate	2·72	2·43	−0·29	0·047	92·2
2. Inf. allant. fl.	2·96	2·86	−0·10	0·636	
1st eluate	2·81	2·75	−0·06	0·639	
2nd eluate	2·68	2·68	0·00	0·043	93·2
3. Inf. allant. fl.	2·75	2·74	−0·01	0·557	
1st eluate	2·63	2·68	0·05	0·560	
2nd eluate	2·41	2·48	0·07	0·028	95·0
4. Inf. allant. fl.	2·71	2·81	0·10	0·574	
1st eluate	2·59	2·71	0·12	0·574	
2nd eluate	2·54	2·66	0·12	0·027	95·3
5. Inf. allant. fl.	2·68	2·76	0·08	0·563	
1st eluate	2·60	2·68	0·08	0·551	
2nd eluate	2·43	2·51	0·08	0·043	92·4
6. Inf. allant. fl.	2·68	2·68	0·00	0·515	
1st eluate	2·41	2·51	0·10	0·543	
2nd eluate	2·38	2·38	0·00	0·044	91·5
7. Inf. allant. fl.	2·68	2·68	0·00	0·568	
1st eluate	2·28	2·38	0·10	0·599	
2nd eluate	2·11	2·23	0·12	0·030	94·7
8. Inf. allant. fl.	2·38	2·70	0·32	0·629	
1st eluate	2·36	2·60	0·24	0·647	
2nd eluate	2·11	2·45	0·34	0·031	95·1
9. Inf. allant. fl.	2·93	2·81	−0·12	0·679	
1st eluate	2·72	2·75	0·03	0·708	
2nd eluate	2·60	2·58	−0·02	0·042	93·8
10. Inf. allant. fl.	2·72	2·71	−0·01	0·459	
1st eluate	2·68	2·68	0·00	0·462	
2nd eluate	2·60	2·34	−0·26	0·040	91·3

* All titres are expressed in $\log_{10}$ units.

† Calculated according to the formula $100 \times \frac{\text{N of I.A.F.} - \text{N of 2nd Eluate}}{\text{N of I.A.F.}}$.

TABLE II.*—*Correlation of Enzymic Activity with Haemagglutinin Titre. Effect of Purification by Adsorption of the Virus on Calcium Phosphate.*

No.	Enz. titre (*a*).	HA titre (*b*).	b − a.	mg. N/ml.	Purification as p.c. N removed.†
1. Inf. allant. fl.	2·51	2·68	0·17	0·658	
Purified virus	2·93	3·06	0·13	0·009	98·6
2. Inf. allant. fl.	2·63	2·68	0·05	0·568	
Purified virus	2·81	2·68	−0·13	0·011	98·1
3. Inf. allant. fl.	2·64	2·71	0·07	0·487	
Purified virus	3·03	3·23	0·20	0·008	98·4
4. Inf. allant. fl.	2·66	2·71	0·05	0·540	
Purified virus	2·68	2·78	0·10	0·011	98·0
5. Inf. allant. fl.	2·98	2·63	−0·35	0·448	
Purified virus	3·23	2·98	−0·25	0·008	98·2
6. Inf. allant. fl.	2·96	2·90	−0·06	0·540	
Purified virus	3·05	2·98	−0·07	0·013	97·6

* cf. Table I. † cf. Table I.

TABLE III.*—*Correlation of Enzymic Activity with Haemagglutinin Titre. Effect of Purification by Adsorption and Elution of the Virus followed by High-speed Centrifugation.*

No.	Enz. titre (*a*).	HA titre (*b*).	b − a.	mg. N/ml.	Purification as p.c. N removed.†
1. Inf. allant. fl.	2·60	2·68	0·08	0·445	
Purified virus	2·08	2·20	0·12	0·008	98·2
2. Inf. allant. fl.	3·02	2·72	−0·30	0·774	
Purified virus	2·30	1·78	−0·52	0·019	97·5
3. Inf. allant. fl.	2·72	2·92	0·20	0·495	
Purified virus	1·88	2·20	0·32	0·009	98·2
4. Inf. allant. fl.	2·68	2·81	0·13	0·518	
Purified virus	2·08	2·41	0·33	0·006	98·8
5. Inf. allant. fl.	2·62	2·71	0·09	0·603	
Purified virus	2·04	2·26	0·22	0·006	99·0

* cf. Table I. † cf. Table I.

TABLE IV.—*Statistical Evaluation of Tables I, II and III.*

	Series I.			Series II.		Series III.	
	Infected allantoic fluid.	1st eluate.	2nd eluate.	Infected allantoic fluid.	Purified virus.	Infected allantoic fluid.	Purified virus.
Mean ($\bar{x}$)*	0·004	0·053	0·016	−0·012	−0·003	0·040	0·094
Standard error of the mean difference†	0·031			0·045		0·073	

* $\bar{x} = \frac{\Sigma x}{n}$; $x = \log_{10} \frac{\text{HA titre}}{\text{Enz. titre}}$.

† The standard error of the mean difference was obtained from the error mean square of the analysis of variance into contributions from experiments, purifications and error (see R. A. Fisher, 1949).

were firmly adsorbed to rather than integral parts of the virus particle. The contrasting fact regarding the enzymic activity of the influenza virus is that in this case there is no indication of the presence in the host cell of the enzyme concerned. As reported previously (Gottschalk and Lind, 1949*b*) no enzyme similar to that associated with the influenza virus could be detected in the normal allantoic fluid from the embryonated egg or in extracts of the ground chorio-allantoic membrane, the host tissue of influenza virus. Moreover, Edney and Isaacs (1950) have shown that the cells of the chorio-allantoic membrane contain, as normal constituent, an intracellular receptor inhibiting haemagglutination by influenza virus; upon infection of the membrane (*in situ*) with influenza virus by the allantoic route the inhibitor is reduced by about 90 per cent. The intracellular inhibitor remains almost undiminished after thorough grinding of the chorio-allantoic membrane with sand, and incubation at 37° for 18 hours, thus ruling out the possibility of the presence in the host cell of an enzyme capable of acting on the inhibitor, but prevented from doing so in the structurally intact cell by spatial separation from its substrate.

It would thus appear that the accumulated evidence is incompatible with the view that the enzyme present in the virus-infected allantoic fluid and in the purified virus preparations is only a contaminant pre-existing in the host cells. The available data are, however, perfectly consistent with the concept that the enzyme, like other virus components, is produced by the host cell—under the changed control of the cell metabolism enforced by the invading and propagating virus—and incorporated into the virus particle. We conclude, therefore, that the enzymic function of the influenza virus resides in some specific grouping arranged at the surface of and representing an integral part of the virus particle.

SUMMARY.

By three independent procedures of influenza virus purification, removing up to 99 per cent of the nitrogen of the crude virus preparation, the ratio haemagglutinin titre/enzymic activity was found to be not significantly changed. It was further observed that the inhibitor of virus haemagglutination shown by Edney and Isaacs to be present inside the normal host cell (chorio-allantoic membrane) and to be destroyed upon virus infection, survives, practically undiminished, thorough grinding of the membrane and incubation at 37° for 18 hours. From these results it is concluded that the enzyme associated with the influenza virus is an integral part of the virus particle, not pre-existing in the normal host cell.

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OBSERVATIONS ON THE ANTIGENIC RELATIONSHIP BETWEEN THE VIRUS OF ENZOOTIC ABORTION IN EWES AND VIRUSES OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP.

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RECENTLY Stamp and his co-workers (Stamp, McEwen, Watt and Nisbet, 1950) demonstrated a virus in the foetal membranes of ewes, and showed that it was responsible for cases of abortion and premature lambing in these animals. The infection is known as enzootic abortion of ewes (E.A.E.).

In its morphology and staining reactions the virus showed a resemblance to those of the psittacosis-lymphogranuloma group and could readily be grown in embryonated eggs. Preliminary investigations in this laboratory (Barwell and Bishop, 1951), using heated yolk-sac suspensions of E.A.E. virus in complement-fixation tests, showed that it will cross-react to titre with human sera from cases of psittacosis and lymphogranuloma venereum (L.G.V.). It was, therefore, considered desirable to investigate more fully the antigenic relationship between these three viruses, and this paper records the results of complement-fixation tests using absorbed and unabsorbed sera.

MATERIALS AND METHODS.

Virus strains.

The E.A.E. strain was that isolated by Stamp *et al.* (1950). Psittacosis virus, strain MOH. 154, was isolated by Professor S. P. Bedson in 1938 from a parrot. The J.H. strain of L.G.V. virus was supplied by Dr. G. Rake.

Sera.

Antisera to E.A.E. virus came from experimentally infected ewes and were kindly sent by Mr. Stamp ; some were also prepared in this laboratory by immunizing guinea-pigs. Human sera from cases of psittacosis and L.G.V. were used.

Antigens.

Yolk-sacs of developing hens' eggs inoculated between the 4th and 6th day of incubation were harvested as soon as most of them were found to be dead. This time, in the case of psittacosis virus, was between 2 and 3 days, and for the other two about 5 days. Each yolk-sac was placed in 2 ml. of M/50 phosphate buffer at pH 7·6 and allowed to stand overnight at 4°. Much of the virus is liberated into the hypotonic solution. The suspension was clarified by light centrifugation and pooled. The virus was then deposited by centrifuging for 1½ to 2 hours in an angle centrifuge at 4500 r.p.m. and was re-suspended in saline,

usually to half the original volume in the case of L.G.V. virus and to the full volume in the case of psittacosis and E.A.E. viruses. The amount of saline used depended on the quantity of virus seen in smears stained by Castaneda's method. If time permitted, the suspension was left overnight in the refrigerator so as to allow flocculation and removal of some extraneous material. The antigen prepared by this method appeared to be at least as good as that made in the usual way after grinding up the yolk-sacs in TenBroeck tubes ; rather it appeared to be purer in that it contained much less cell débris. Antigens were prepared from infected mouse lung or mouse spleen, as described above, from suspensions made by grinding the tissue in buffer by means of a TenBroeck tube. These were used in testing guinea-pig sera which, after immunization, contained antibodies to yolk-sac. Unheated antigens were used without preservative and as fresh as possible. For use as heated antigens, suspensions were placed in a boiling water-bath for 20 minutes, sodium azide was added to 0·3 per cent as a preservative and the product was finally titrated against dilutions of a known positive serum.

Complement.

Pooled guinea-pig serum stored in a dry ice unit was used. It was titrated on the day of the test and 2 M.H.D. was used in all tests.

The test.

This was done in the manner described by Bedson, Barwell, King and Bishop (1949). Appropriate doubling dilutions of serum, 2 M.H.D. of complement and an optimal dilution of the antigen, each in 0·1 ml. volume, were mixed and, after the addition of 0·5 ml. of saline, allowed to react at room temperature for $\frac{1}{2}$ hour and at 37° for $\frac{1}{2}$ hour. Serum controls and normal tissue antigen controls were included. Sheep cells, 0·2 ml. of a 5 per cent suspension sensitized with 5 M.H.D. of amboceptor, were added to each tube, and readings were made after incubation at 37° for $\frac{1}{2}$ hour and again after standing overnight at room temperature. Complete fixation was recorded as ++++, and degrees of partial fixation by a smaller number of + signs roughly indicating the amount of cells not haemolysed.

Absorption of sera.

Serum at a concentration 8 times that at which it showed definite but partial complement-fixation with the heated antigen was absorbed with the washed deposit from an equal volume of the virus suspension. After standing overnight in the refrigerator the absorbed serum was separated by angle centrifugation for 2 hours at 7000 r.p.m.

RESULTS.

Boiled or unheated suspensions of the three viruses were used to absorb in turn the different antisera studied. When the absorbed sera were tested against various heated and unheated antigens it was found in general that absorption with heated virus gave satisfactory and consistent results while those obtained with unheated virus were irregular.

Absorption with boiled virus.

Tables I and II show examples of absorption with heated, heterologous virus suspensions. In the first of these experiments serum from E.A.E. infected sheep 776 was tested, unabsorbed and after absorption with boiled psittacosis virus, against both boiled and fresh E.A.E. and psittacosis virus preparations. Absorption removed the antibody against both heated suspensions more or less completely and against the unheated heterologous virus. The absorbed serum still contained an antibody giving complement-fixation with the unheated homologous virus. It will be seen that the unheated antigens were used at twice the concentration of the boiled preparations and even then usually failed to react as strongly. The same results as those shown in Table I were obtained when heated E.A.E.

TABLE I.—*Absorption of E.A.E. Sheep Serum with Heated Psittacosis Virus.*

Serum.		Antigens + 2 M.H.D. complement.					
		Heated (100°) 1 in 4.		Unheated 1 in 2.		Normal yolk-sac 1 in 2.	Saline.
Name.	Dilution.	Psitta-cosis.	E.A.E.	Psitta-cosis.	E.A.E.		
E.A.E. serum 776, unabsorbed	1/8	++++ / ++++	++++ / ++++	+++ / ++	++++ / +++	− / −	− / −
	1/16	++++ / ++++	++++ / ++++	+++ / ++	++++ / +++		
	1/32	++++ / ++++	++++ / ++++	+++ / ++	++++ / +++		
	1/64	+++ / +++	+++ / +++	++ / ++	+++ / ++		
E.A.E. 776, absorbed with heated psittacosis virus	1/8	− / −	+ / ±	− / −	+++ / +++	− / −	− / −
	1/16	− / −	± / −	− / −	+++ / ++		
	1/32	− / −	− / −	− / −	+++ / ++		
Pooled normal guinea-pig serum	1/4	− / −	− / −	− / −	− / −	− / −	− / −
Saline		− / −	− / −	− / −	− / −	− / −	− / −

Readings after 30 min. at 37° (upper row) and after standing overnight at room temperature (lower row).

virus was used for absorption. Similarly, absorption of human sera from cases of L.G.V. (Table II) or psittacosis with heated E.A.E. virus removed antibody to all heated suspensions and to unheated heterologous virus preparations, while fixation with fresh homologous virus was only slightly reduced. Except in so far as different batches of fresh partially purified suspensions of the same virus vary in their complement-fixing potency, the results obtained by absorbing a given serum with any of these heated virus preparations were consistently the

same. Whatever combination of serum and heated virus was tested for absorption, antibody only to the unheated homologous suspension was left.

Absorption with unheated virus.

Absorption with the different unheated virus suspensions has, however, been found to give somewhat irregular and unpredictable results. Examples of the findings in some of these experiments are given in the next two tables, but it

TABLE II.—*Absorption of Human L.G.V. Serum with Heated E.A.E. Virus.*

Serum.		Antigens + 2 M.H.D. complement.				
		Heated (100°) 1 in 4.	Unheated 1 in 2.		Normal yolk-sac 1 in 2.	Saline.
Name.	Dilution.	L.G.V.	L.G.V.	E.A.E.		
L.G.V. serum P. unabsorbed	1/16	++++ . ++++	++++ . ++++	++++ . ++++	− . −	−
	1/32	++++ . ++++	++++ . ++++	++++ . ++++		
	1/64	++++ . ++++	+++ . +++	+++ . +++		
L.G.V. serum P. absorbed with heated E.A.E. virus	1/16	++ . ++	++++ . ++++	+ . ±	− . −	−
	1/32	± . −	+++ . ++	− . −		
	1/64	− . −	± . −	− . −		
Normal human serum	1/4	− . −	− . −	− . −	− . −	−
Saline		− . −	− . −	− . −	− . −	−

must be emphasized that these illustrate results which are by no means consistent. In Table III E.A.E. sheep serum 771 has been absorbed with unheated psittacosis virus, and slight but equal reduction of fixing power for the heated and for the two unheated suspensions has resulted. At other times heterologous absorption of this kind effectively removes the group antibody, and leaves the specific one as shown in Table IV in the columns referring to the behaviour of serum P. (L.G.V.) after treatment with unheated E.A.E. or psittacosis viruses. These two tables also show the results which may be obtained when a serum is absorbed with the unheated homologous virus. The usual effect is that set out in Table III, where both types of antibody have been completely or almost completely removed (Bedson, 1936). In Table IV the homologous, unheated virus, which was expected to absorb both antibodies from serum P., has only partially reduced the group antibody, while the specific antibody seems to have been more selectively absorbed.

DISCUSSION.

The results consistently obtained by absorbing sera with heated virus provide further support for the idea that these agents possess at least two antigens, one heat-

resistant and group-reactive, the other heat-labile and specific (Bedson, 1936; Barwell, 1948; Bedson *et al.*, 1949). The direct complement-fixation and cross-absorption experiments clearly show that E.A.E. virus possesses the heat-stable group antigen in common with the viruses of the psittacosis-lymphogranuloma group, and that it too has a specific component which is destroyed by heat. Absorption of sera with unheated virus gives irregular and unpredictable results.

TABLE III.—*Absorption of E.A.E. Sheep Serum with Unheated E.A.E. and Psittacosis Viruses.*

Serum.		Antigens + 2 M.H.D. complement.					
		Heated (100°) 1 in 4.		Unheated 1 in 2.		Normal yolk-sac 1 in 2.	Saline.
Name.	Dilution.	E.A.E.	Psittacosis.	E.A.E.	Psittacosis.		
E.A.E. serum 771, unabsorbed	1/8	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + +	− . −	− . −
	1/16	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + +		
	1/32	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +	+ + + . + + +		
	1/64	+ + + + . + + +	+ + + + . + + +	+ + + + . + + +	+ + + . + + +		
E.A.E. 771, absorbed with unheated psittacosis virus	1/8	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +	− . −	− . −
	1/16	+ + + + . + + +	+ + + . + + +	+ + + + . + + + +	+ + + + . + + + +		
	1/32	− . −	± . −	± . −	± . −		
E.A.E. 771, absorbed with unheated E.A.E. virus	1/8	− . −	− . −	+ + . +	± . −	− . −	− . −
	1/16	− . −	− . −	± . ±	− . −		
	1/32	− . −	− . −	− . −	− . −		
Normal human serum	1/4	− . −	− . −	− . −	− . −	− . −	− . −
Saline		− . −	− . −	− . −	− . −	− . −	− . −

Using the homologous virus the usual result is the removal of both types of antibody, though occasionally this treatment may absorb most of the specific antibody whilst producing only a moderate reduction in the group antibody. Heterologous absorption with unheated virus in some experiments had the effect of reducing partially and to a comparable extent the titre for heated and unheated virus suspensions, whereas at other times there was a selective and more or less complete removal of group antibody. These irregular effects of absorbing with

fresh virus preparations are not necessarily at variance with the concept of two antigenic components in these viruses. Experience in this laboratory suggests that the serological activity of the specific antigen is much the weaker of the two ; and it is for this reason that the fresh suspensions are used as antigens at a higher concentration than the heated ones. However fresh the virus preparation, it is impossible to predict with certainty that the specific antigen will be present in a fully serologically reactive state. Furthermore it is labile, and preparations which when first prepared reacted specifically to full titre may lose this character on storage at 4° in three or four weeks' time. This deterioration of the specific antigen may or may not be accompanied by enhanced activity of the group

TABLE IV.—*Absorption of Human L.G.V. Serum with Unheated Homologous and with Two Unheated Heterologous Viruses.*

Serum.		Antigens + 2 M.H.D. complement.					
		Heated 100° 1 in 4.	Unheated 1 in 2.			Normal yolk-sac 1 in 2.	Saline.
Name.	Dilution.	Psittacosis.	L.G.V.	E.A.E.	Psittacosis.		
L.G.V. serum P., unabsorbed	1/16	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +	— . —	— . —
	1/32	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +	+ + + + . + + + +		
	1/64	+ + + + . + + + +	+ + + + . + + +	+ + + + . + + +	+ + + + . + + + +		
L.G.V. serum P., absorbed with unheated L.G.V. virus	1/16	+ + + + . + + + +	+ + . + +	+ + + + . + + + +	+ + + + . + + + +	— . —	— . —
	1/32	+ + + + . + + + +	± . —	+ + . + +	+ + + . + + +		
	1/64	± . —	— . —	— . —	± . —		
L.G.V. serum P., absorbed with unheated E.A.E. virus	1/16	+ . +	+ + + + . + + + +	— . —	+ . +	— . —	— . —
	1/32	— . —	+ + + + . + + +	— . —	— . —		
	1/64	— . —	+ + . +	— . —	— . —		
L.G.V. serum P., absorbed with unheated psittacosis virus	1/16	— . —	+ + + + . + + + +	— . —	— . —	— . —	— . —
	1/32	— . —	+ + + . + + +	— . —	— . —		
	1/64	— . —	+ + . +	— . —	— . —		
Normal human serum	1/16	— . —	— . —	— . —	— . —	— . —	— . —
Saline		— . —	— . —	— . —	— . —	— . —	— . —

TABLE V.—*Human L.G.V. Serum Tested at Intervals against Unheated or Boiled Fractions of an L.G.V. Suspension.*

L.G.V. antigen J.H. 15. Prepared 23.vii.47. Diluted 1 in 4.

Serum. Name.	Dilution.	Unheated. Tested on— 1.viii.47.	15.viii.47.	19.viii.47.	Heated at 100° on— 23.vii.47. Tested on— 1.viii.47.	15.viii.47.	19.viii.47.	19.viii.47. Tested on— 19.viii.47.	Normal yolk-sac.	Saline.
L.G.V. serum GRE.	1/4	++++	+++	++	++++	++++	++++	++++	−	−
		++++	++	+	++++	++++	++++	++++	−	−
	1/8	++++	+	+	++++	++++	++++	++++		
		++++	±	±	++++	++++	++++	++++		
	1/16	++++	+	∓	++++	++++	++++	++++		
		++++	±	−	++++	++++	++++	++++		
	1/32	++++	±	−	++++	++++	++++	++++		
		++++	∓	−	++++	++++	++++	++++		
	1/64	++++	∓	−	++++	++++	++++	++++		
		+++	−	−	++++	++++	++++	++++		
Normal human serum	1/4	−	−	−	−	−	−	−	−	−
		−	−	−	−	−	−	−	−	−
Saline		−	−	−	−	−	−	−	−	−
		−	−	−	−	−	−	−	−	−

antigen. In other words the labile component may have little or no serological activity, while still being capable of interfering with the group antigen. This phenomenon was observed by Bedson (1936) with psittacosis virus, and that the same thing may occur with L.G.V. virus is shown by the findings presented in Table V. The explanation of this is not known, but it could be accounted for by some qualitative change in the specific antigen itself. The possibility that absorption of tissue components present in the suspension may play a part has not been excluded.

It appears, however, that fresh virus may react with sera containing both types of antibody in one of three ways. Both specific and group antigens may be available and react with their respective antibodies, and this is what happens most commonly. Sometimes the fresh virus behaves mainly as a specific antigen, and on other occasions it may have little or no activity unless heated when, of course, it behaves as a group antigen. In view of this it is not surprising that the results of absorbing sera with unheated viruses of this group tend to be unpredictable, and further work on the serological behaviour of these agents is required to gain a further insight into their antigenic complexity. However, this in no way detracts from the use which can be made of sera absorbed with heated virus in the study of unidentified viruses belonging to this group, and this technique can enhance the value of tests on patients' sera.

SUMMARY.

The virus of enzootic abortion in ewes has been studied in complement-fixation tests using as antigens, unheated and boiled virus suspensions.

Sera against psittacosis and lymphogranuloma venereum viruses and similar preparations of these agents were used in cross-absorption experiments.

The sheep virus was found to possess the heat-stable group antigen common to the group as well as a heat-labile, specific component.

The antigenic behaviour and relationships of these viruses are discussed.

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SPONTANEOUS MUTATION OF INFLUENZA VIRUS A DURING ROUTINE EGG PASSAGE.

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THE influenza viruses are notoriously labile; it is their lability which probably accounts for the multiplicity of strains showing minor antigenic differences and also for their comparatively ready adaptability to new host species. The establishment of a new variant is commonly believed to be due to the selective action of environmental conditions upon a chance mutant which happens to be favoured by that particular environment. The literature contains many examples of variant strains probably produced by such a mechanism in which change of host species or modifications of the method or route of inoculation has been involved, but we know of no recorded instance of a well adapted, stable strain being replaced by a variant during rigorously controlled routine passage (Briody, 1950). The present paper reports such an occurrence, and by a comparison between the variant and the parent strains provides evidence that mutation of the influenza viruses may occur spontaneously with effects upon important biological activities, but without necessarily affecting antigenic constitution.

MATERIALS AND METHODS.

Virus strains.

The parent strain was an egg-adapted PR8 strain of Influenza A virus obtained from the National Institute for Medical Research in 1947. Its exact history up to that time is unknown, but, prior to the appearance of the variant, it had undergone 80 consecutive allantoic passages in this laboratory without any detectable change. Subsequent to the occurrence of mutation the parent strain was resuscitated from stored material and maintained by further serial passages in eggs without any recurrence of deviation from its characteristic behaviour. It is referred to as "PR8/Stock."

The mutant strain is referred to as PR8/X. It became established as the dominant virus at about the 80th serial passage of the parent, since when it has been maintained by continued serial allantoic egg passages without exhibiting any further change.

Virus preparations.

For most experiments virus in the form of infected allantoic fluid was used. Fertile hen eggs at the 10th day of incubation were inoculated into the allantoic

* Seconded for virus research from the staff of the Public Health Laboratory Service.

cavity with 0·2 ml. of diluted allantoic fluid from a previous passage, the dilution, usually $1/10^3$ to $1/10^5$, being chosen on the basis of haemagglutination titre. Eggs were incubated at 36–37° for 48 hours. They were chilled overnight at 4° and the fluids harvested. Fluids from each batch were usually pooled, lightly centrifuged, titrated for haemagglutination titre and either used immediately or stored until required at 4°.

For some experiments partially purified and concentrated virus suspensions were prepared from pooled allantoic fluids by adsorption on human group O red blood cells and subsequent elution.

Haemagglutination.

Titrations were carried out by a modified photoelectric densitometer method (Belyavin, Westwood, Please and Smith, 1951). This allowed not only a precise numerical determination of the end-point, but also accurate assessment of the significance of results. Haemagglutinin inhibition tests were invariably made against 4 agglutinating doses of virus, and serum titres are expressed as reciprocals of the final serum dilutions at the 50 per cent agglutination end-point. Fowl cells were used for all titrations.

Complement fixation.

In order that minor differences between strains might be detectable, complement-fixation tests were done with specific virus antigens in the form of allantoic fluids or eluates which were devoid of the soluble antigen common to related influenza viruses (Fulton and Dumbell, 1949; Hoyle and Fairbrother, 1937).

For simple titrations constant antigen, standardized by haemagglutinating doses, and doubling dilutions of serum were used. Such tests were soon found to be valid only as a guide in pilot experiments. For crucial experiments chessboard titrations with doubling dilutions of both antigen and antiserum were necessary. Complement was first titrated to the 50 per cent haemolysis end-point in the presence of the largest dose of antigen to be used. The indicator system was a 1 per cent suspension of sensitized sheep red cells. In the test proper 2 units of complement were used. Fixation time was 30 minutes at 37°. A further 30-minute period of incubation at 37° was allowed after addition of the red cell suspension, after which racks were transferred to the ice-box overnight. Readings were taken on the photo-electric densitometer, which permitted numerical evaluation of all degrees of haemolysis.

EXPERIMENTAL.

Origin and Detection of the Mutant.

During the course of an investigation on the mechanism of the Francis phenomenon, PR8 stock virus was used almost daily in titrations of serum inhibitors (Francis, 1947). It was chosen for this work because of its sensitivity to such inhibition. The inhibitory titres of normal guinea-pig sera when tested against 4 A.D. living virus varied between 100 and 300, whilst against 4 A.D. of heated virus (56° 30 minutes) the titres ranged from 300 to several thousands. Corresponding figures for normal rabbit sera were 30–80 and 160–3000. Thus the virus was readily converted to the indicator state by heat treatment. As there

is some confusion in the literature as to what is meant by the indicator state, it is proposed to use the term throughout the present paper to denote an increased susceptibility of any virus to the inhibitory action of serum haemagglutinin inhibitors occurring as the result of its degradation by heat treatment or other means. The behaviour of PR8 in this respect remained quite constant up to about the 80th allantoic passage, at which point the titres of both guinea-pig and rabbit sera against living virus showed a puzzling fall whilst satisfactory indicator virus could no longer be produced by heat treatment. It is not possible to designate the exact passage at which the change occurred, because virus batches from passages 77, 78 and 79 were not used in such tests, but the anomalous behaviour was certainly fully established at the 83rd passage, and has continued without further modification up to the current 98th passage. Resuscitation of the parent virus from freeze-dried material of the 35th passage and its serial propagation in parallel with the main line has yielded no evidence of any repetition of the phenomenon. There can thus be little doubt that a naturally occurring mutant was able to supplant the parent virus during routine allantoic passage under carefully standardized conditions. The transition is illustrated by the results of a number of serum titrations summarized in Table I.

TABLE I.—*Normal Serum Titres Illustrating Transition of PR8 Virus from Normal to Mutant Behaviour.*

	Serum titres against 4 A.D.'s of virus.											
Virus passage No.:	74.		76.		80.		81.		83.	
	Living.	Heated.	Living.	Heated.	Living.	Heated.	Living.	Heated.	Living.	Heated.
Normal guinea-pig serum	136	691	77	296	—	—	<40	475	<20	40
	160	788	86	320	—	—	45	545	<20	45
Normal rabbit serum	27	113	28	70	—	—	<20	80	<20	34
	80	1076	97	134	—	—	<20	98	21	40
Normal human serum	297	877	—	—	61	104	—	—	55	58
	80	269	—	—	110	368	—	—	59	75

Different samples of normal sera were used against the different passage batches of virus.

Antigenic Structure of the Mutant.

Exclusion of laboratory contamination.

Our immediate reaction to this sudden change of behaviour was to suspect contamination with some other virus strain, for many cases of laboratory contaminants being isolated as new viruses are on record (Andrewes, Glover, Himmelweit and Smith, 1944). Three other influenza viruses were in use in the laboratory at the time: an A prime strain (Dutch), a B virus (Lee), and a Swine virus (SW15). The antigenic differences between each of these and PR8 are so clearly marked that complete cross neutralization tests were not considered necessary. It sufficed to compare the behaviour of PR8/stock and PR8/X viruses again each specific antiserum in turn in a number of haemagglutination inhibition tests. The results are given in Table II. Lee B and SW antisera showed no neutralization of either virus strain. The A prime antiserum, as was to be expected, exhibited considerable potency against PR8/stock, but its very much lower titre against PR8/X removed the suspicion that the latter might be

TABLE II.—*Haemagglutination Inhibition Titres of Type Antisera against PR8/S and PR8/X Viruses.*

Expt. No.	Immune sera.	Virus. PR8/stock.	PR8/X.
1	PR8	266	200
2	PR8	422	232
	A prime (Dutch)	320	59
3	PR8	282	202
	B (Lee)	<40	<40
4	PR8	364	215
	Swine (SW 15)	<40	<40

an A prime (Dutch) contaminant virus. In fact the titre difference merely reflects the anomalous behaviour of PR8/X against normal serum inhibitors, and is similarly shown with the PR8 immune serum. Laboratory contamination with any of the strains in current use can thus be ruled out as an explanation of the sudden change of behaviour of the PR8 passage virus.

Antigenic analysis by haemagglutination inhibition tests.

The significantly lower titres of both PR8 and A prime immune sera against the mutant strain suggested that the mutation might have involved an alteration of antigenic structure, and called for a more thorough analysis of the antigenic relationship between parent and derivative strains. Specific immune sera to each strain were therefore prepared in rabbits by single intraperitoneal injections of infected allantoic fluids. Cross-haemagglutination inhibition tests were then carried out. The results summarized in Table III show that PR8/X was

TABLE III.—*Haemagglutination Inhibition Titres of Specific Antisera against PR8/S and PR8/X Viruses.*

Sera.	Virus. PR8/stock.	PR8/X.
R9 Anti-X	2300	861
R10 Anti-X	1208	896
R9 Anti-X	1973	1080
R12 Anti-Stock	4200	1924

uniformly less susceptible to inhibition than PR8/stock by either homologous or heterologous serum, but they provide no evidence of any qualitative antigenic difference. The findings however, are quite consistent with the hypothesis that the X mutant represents either a quantitative antigenic change or a change affecting the spatial arrangement of the virus surface antigens.

Complement-fixation tests.

We have obtained clear evidence in work not yet published that surface antigens of the influenza viruses, other than the haemagglutinin components, may participate in the complement-fixation reaction. Thus haemagglutination

inhibition titres and complement-fixation titres of immune sera, although often showing a close parallelism, need not necessarily do so. A number of simple complement-fixation tests with the specific rabbit immune sera against constant antigen dose standardized on the basis of the virus haemagglutination activity were carried out. They revealed no antigenic difference between stock and mutant strains. Finally a full chessboard analysis was made, the results of which are illustrated in Fig. 1. Determination of all degrees of haemolysis is possible by means of the photoelectric densitometer, so that the complement-fixing potency of a virus can be charted as an area of fixation with considerable

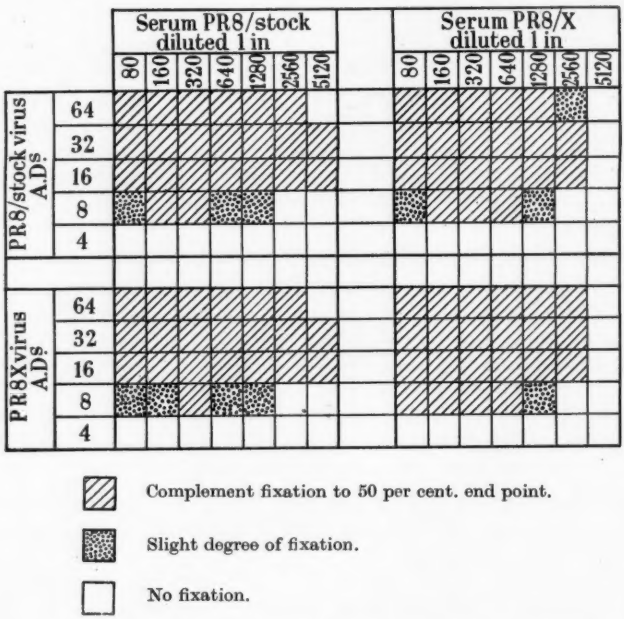

FIG. 1.—Comparison of stock and X strains by complement-fixation.

accuracy. Stock and mutant strains against the homologous and heterologous sera respectively gave fixation areas so nearly identical that antigenic identity of the two viruses is extremely probable, at least as far as complement-fixing antigens are concerned. This in itself is good evidence that PR8/X is a mutant derived from PR8/stock during routine passage of the latter.

Characters of the Mutant Strain.

Relative insusceptibility to serum inhibitors.

The property of PR8/X which first drew attention to the possibility of mutation was its relative insusceptibility, whether in the living or heat-degraded state, to normal serum inhibitors. The mutant was propagated through a long series

of further consecutive allantoic passages in eggs, and the parent strain, resuscitated from material which had been freeze-dried long before the occurrence of mutation, was propagated through a parallel passage series with identical technique. Corresponding batches of the two viruses, both living and heated at 56° for 45 minutes, were tested in parallel against many samples of sera obtained from man, rabbit and guinea-pig. Virus-serum mixtures were allowed 30 minutes' contact at room temperature before the addition of fowl red cells. The striking and consistent difference of behaviour between the two virus strains is shown by the results summarized in Table IV. If, as we have reason to believe, there are qualitative differences between the normal serum inhibitors of different animal species, it is clear that the mutational resistance of X is equally effective against each type of inhibitor.

The thermolabilities of the two strains were examined by comparing the percentage reductions of haemagglutinating activity when they were heated side by side in a water-bath at 56°. In a series of 26 direct comparisons with periods of heat treatment from 20 minutes to 65 minutes there was great variability of thermolability with different batches of allantoic fluid in the case of each virus. PR8/stock, however, suffered the greater percentage reduction in 20 experiments and in only 6 experiments was the position reversed. Statistical analysis of the total results showed a highly significant difference between the mean percentage reductions of titre, indicating clearly that the mutant strain possesses greater heat stability. The factor in allantoic fluid which may occasionally outweigh the difference of stability of the viruses themselves is unknown.

Further experiments were designed to investigate the time relations of the Francis responses. Each virus was distributed in ampoules which were immersed in a water-bath at 56°. An ampoule of each was removed at selected time intervals, cooled rapidly and the viruses titrated. Serum inhibitor titrations were then carried out against 4 A.D. of each virus sample. Fig. 2 illustrates the types of curve obtained in a number of such experiments.

Invariably virus X proved to be less sensitive to serum inhibitor than stock virus. Its sensitivity was certainly increased by continued heat treatment in conformity with previous findings of Smith and Westwood (1950), but at no time throughout the period of experiment was its sensitivity as great as that of stock virus at the corresponding time. Apart from irregularities during the first hour, both strains showed smooth sensitivity increase as long as sufficient virus activity remained to permit the use of 4 A.D. in a reasonable volume. This initial irregularity has been too constant a phenomenon to be dismissed as being due to experimental error, but its cause has not yet been elucidated.

The effect of ether treatment.

During work not yet published on the effects of treating influenza viruses with ether it was found that PR8/S was converted into the indicator state just as readily as by heat treatment but without appreciable destruction of its enzymic activity. Somewhat surprisingly in view of its failure to be converted to an indicator virus by heat the X mutant was found to respond to ether treatment in a similar manner.

Enzymic activity was measured by the percentage recovery of virus by elution from red cells to which it had previously been adsorbed. It will be seen from the experiments recorded in Table V that even the heated virus possessed

TABLE IV.—*Inhibitor Titrations of Various Sera against Stock and Mutant Strains, Living and Heated.*

Sera. Species.	Number tested.	Virus.		Haemagglutination inhibition titres.								
Human	6	Stock/A1 195	L	202	243	135	298	75	>320	—	—	—
			H	>2560	>2560	>2560	>2560	>2560	>2560	—	—	—
		X/A1 196	L	104	160	49	198	46	>320	—	—	—
			H	127	160	67	145	64	>320	—	—	—
Rabbit	9	Stock/A1 193	L	25	48	57	25	20	110	46	<20	34
			H	971	2077	1687	1372	640	>2560	>2560	360	538
		X/A1 194	L	<20	25	<20	<20	<20	34	33	<20	24
			H	38	65	72	74	49	160	109	57	32
Guinea-pig	5	Stock/A1 193	L	96	61	73	69	80	—	—	—	—
			H	3879	2727	5120	3879	>10,240	—	—	—	—
		X/A1 194	L	40	40	44	37	63	—	—	—	—
			H	87	87	76	91	320	—	—	—	—

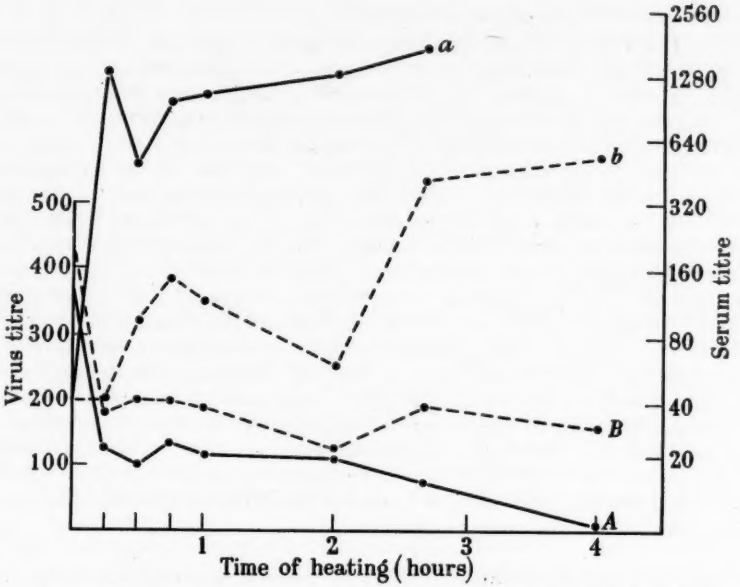

FIG. 2.—Time relations of the Francis responses given by Stock and X strains.

A = Titres of Stock virus.
B = „ X virus.
a = Serum inhibitor titres against Stock virus.
b = „ „ „ „ X virus.

TABLE V.—*Effect of Heat and Ether Treatment on Enzyme Activity and Inhibitor Sensitivity of the PR8/X Mutant.*

Expt.	PR8/X virus.	Titre.	Titre of eluate.	Per cent recovery.	Inhibition titre of normal rabbit serum.
1	Living	1130	—	—	<40
	Heated	577	175	30	<40
	Ether treated	941	625	66·5	160
2	Living	2100	—	—	<40
	Heated	1170	320	27	<40
	Ether treated	996	862	87	320

some residual enzymic activity, but this was very much less than that of the ether-treated samples. Indeed enzymic loss from ether treatment in Experiment 2 must have been negligible, because in the 3 hours allowed for elution 100 per cent recovery is never obtained even with a fresh living virus. In both experiments the inhibitor titres of normal rabbit serum provide good illustrations of the Francis phenomenon. The X mutant thus yielded incontrovertible proof that the Francis phenomenon does not depend upon inactivation of the virus enzyme which destroys normal serum inhibitors (Smith and Westwood, 1950).

Mouse virulence and protection experiments.

Both viruses were found to possess a surprisingly high mouse virulence. Allantoic fluids with haemagglutination titres of between 200 and 400 regularly produced lung lesions in Swiss mice of 18–20 g. weight at a dilution of 10^{-5} or 10^{-6}. Such lesions caused by egg virus prior to mouse adaptation are a reflection of virus toxicity rather than of infectivity, but the term " virulence " conveniently covers the summation of both types of virus activity. In this respect there appeared to be no difference between the stock and X strains.

In view of the lesser sensitivity of the X mutant to inhibition by normal sera in haemagglutination tests, it was thought possible that it might also be less sensitive to neutralization in mouse neutralization tests. Smith and Westwood (1949) reported that as many as 1000 infective doses of PR8 virus might be neutralized by a fresh guinea-pig serum, and considered that the effect was probably due to the high haemagglutinin inhibitor content of guinea-pig sera. Ten-fold dilutions of each virus from 10^{-2} to 10^{-6} were mixed with equal volumes of the pooled serum from six guinea-pigs. After 30 minutes' contact at room temperature each mixture was inoculated intranasally into 3 anaesthetized mice. Similar mixtures of virus dilutions and horse serum were used as controls, for Smith and Westwood (1949) showed that horse serum is devoid of detectable neutralizing potency. Approximately 1000 infective doses of stock virus and 100 infective doses of X virus were neutralized.

TABLE VI.—*Mouse Neutralization Tests of Normal Guinea-pig Serum against Stock and X Viruses.*

Serum.	Virus.	Virus dilution ·2 X. 10^{-2}.			10^{-3}.			10^{-4}.			10^{-5}.			10^{-6}.		
Horse	PR8/S	D	D	D	D	D	D	D	+++	+++	+++	++	++	+	+	+
	PR8/X	D	D	D	D	D	D	D	++	++	++	++	0	+	+	+
Guinea-pig	PR8/S	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
	PR8/X	+	+	*	++	+	*	0	0	0	0	0	0	0	0	0

D = Influenzal death with almost complete lung consolidation.
Number of + signs indicates severity of lung involvement in mice killed 6th day.
* = Non-specific death.

DISCUSSION.

Although many instances of mutation of influenza viruses are on record the present case merits attention for several reasons. The fact that the mutant arose during routine egg passage without any modification of technique, and rapidly replaced the parent strain, indicates that such changes may have occurred from time to time without being detected, and thus be responsible for some of the discrepancies and contradictions in which the influenza literature abounds. Had the X mutant developed right at the outset of our work with PR8 virus its behaviour would naturally have been accepted as characteristic of the standard strain supplied to us, and this might well have led to claims in serious disagreement with those of other workers. It is also apparent that a well-established strain may be replaced by one possessed of different biological activities at any stage in the chain of transmission through the natural human host, so that it is not essential to postulate different sources of origin for two or more different viruses which are isolated from a single localized epidemic.

It is, however, in connection with the analysis of virus structure that the phenomenon gains its chief significance. Adaptations to new hosts, or to different tissues of the same host species, are so often accompanied by modifications of antigenic structure that there is some tendency to confine attention to mutations which involve detectable antigenic change, and to overlook the possible importance of structural changes which cannot be demonstrated by the conventional methods of serological analysis. Whilst the possibility of some minor but determinant antigenic change cannot be entirely excluded in the present instance, it is much more probable that the changed behaviour of the virus resulted from heritable modifications of surface configuration, involving particularly those surface receptors concerned in the phenomena of haemagglutination. The X mutant may differ from the parent strain in other ways not so far detected, but its increased heat stability, its lower sensitivity to the haemagglutinin inhibitors of normal sera and to the specific antibodies of immune sera, and its convertibility to the indicator state by ether, but not by the usual method of heat treatment, are all explicable on the assumption that the mutation involved an alteration of the pattern of reactive surface components. A simple rearrangement whereby the haemagglutinin receptors became more widely spaced would suffice to minimize the effects of heat distortion and counteract the steric hindrance postulated by Smith and Westwood (1950) as being a major factor in the mechanism of the Francis phenomenon.

If the haemagglutinin receptors play an important rôle in the process of cell parasitization by virus, as much recent work indicates, it is evident that mutations of the X type without antigenic change may in some instances affect biological characteristics like communicability and virulence, and thus have profound effects on the pattern of influenza epidemics. The sensitivity of a virus to specific antibodies and, perhaps even more, to the neutralizing action of normal serum constituents are also factors which must help to determine the course of a natural outbreak of influenza. It was with this in mind that the preliminary experiments in mice were carried out, but the results of the single mouse neutralization test, though suggestive, indicate that many replicate experiments involving very large numbers of animals will be necessary in order to establish the point at issue.

The retention of almost full enzymic activity by virus after its conversion to the indicator state by ether treatment refutes finally the theory that the Francis phenomenon depends upon heat inactivation of the virus enzyme. Precisely how ether extraction affects so profoundly the quantitative aspects of the receptor inhibitor union is unknown, but the extraction of lipoid components must inevitably cause some distortion of normal surface configuration. The surprising thing is that in the case of the X mutant, heat treatment sufficient to destroy the virus enzyme fails to cause enough distortion for conversion to the indicator state.

SUMMARY.

1. The replacement of an egg passage strain of PR8 influenza virus by a naturally occurring mutant is recorded.

2. The mutant differs from the parent virus in heat stability and haemagglutination characteristics but appears to be antigenically unaltered.

3. The mutant is less sensitive than the parent to haemagglutinin inhibition by either specific antibodies or normal serum inhibitors. It is not converted to the indicator state by the standard method of heat treatment, but it becomes a good indicator virus when extracted with ether in spite of retention of enzymic activity.

4. The implications of virus mutations of this type are discussed.

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THE NUCLEIC ACIDS OF *RICKETTSIA BURNETI*.

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IN recent years much interest has centred about the structure and biological distribution of the nucleic acids. There is evidence to show that these are concerned in protein synthesis and are intimately linked with the genetic mechanism. In a few instances they have been shown to possess specific biological properties, one of the most remarkable being the parts played by deoxypentose nucleic acids in inducing the type transformations of pneumococci and *Bact. coli* (Avery, Macleod and McCarty, 1944; Boivin, Delaunay, Vendrely and Lehoult, 1945). General acceptance of the tetranucleotide hypothesis, originally put forward by Levene (Levene and Bass, 1931), had led to the belief in common structures for all ribonucleic acids (RNA) and all deoxypentose nucleic acids (DNA). Such uniformity of structure was difficult to reconcile with the existence of specific biological properties pertaining to individual nucleic acids. Although Gulland and his co-workers (Gulland, 1947) had thrown considerable doubt on the tetranucleotide hypothesis, proof of its incorrectness had to await the development of new methods, based on chromatography and spectrophotometric analysis, which permitted the accurate analysis of the nucleotide composition of nucleic acids from many sources. In addition to disproving the tetranucleotide hypothesis, the application of these methods has led to the recognition of 5-methyl cytosine as a constituent of DNA from higher animals and plants (Wyatt, 1951*a*), but which is absent in the DNA from bacteria and viruses. It has also been shown that the composition of a deoxypentose nucleic acid is characteristic of the organism from which it is derived (Chargaff, 1950; Wyatt, 1951*b*). This is also true of the ribonucleic acids from the plant viruses, in which it is even possible to demonstrate small differences in composition of ribonucleic acids from different virus strains (Markham and Smith, 1950, and unpublished). These results have stimulated interest in the compositions of nucleic acids from as wide a range of biological sources as possible, and in this paper we describe the analysis of the nucleic acids from a rickettsia, *Rickettsia burneti*.

In size and morphological complexity the rickettsiae occupy a position between bacteria and the larger animal viruses. Chemical analyses of the isolated rickettsial organisms have been limited to *Rickettsia prowazeki*, which has been shown to contain protein, nucleic acid, carbohydrate, lipid and phospholipid. The published quantitative analyses show differences which probably reflect to some extent variations in the procedures used for the purification of the organisms. Cohen and Chargaff (1944) found the dried rickettsiae to contain 13·2 per cent lipids, of which 9·5 per cent was acetone-soluble and 3·1 per cent phospholipids, 12·2 per cent nitrogen and 0·93 per cent phosphorus. The

analyses of Tovarnickij, Krontovskaja and Ceburkina (1946) gave 46·6 per cent lipids (of which 29·7 per cent was neutral fat and 15·8 per cent phospholipids) and only 30·2 per cent protein. The discrepancy between the lipid contents given by the two analyses is almost certainly in part due to the fact that Cohen and Chargaff used ether treatment to remove host material, whereas Tovarnickij and co-workers obtained their preparations entirely by differential centrifugation. Both groups of workers demonstrated nucleic acid in the rickettsiae. By sonic disintegration of the organisms followed by centrifugation and acid precipitation of the supernatant liquid Cohen (1946) isolated nucleic acid amounting to 2–2·5 per cent of the dry weight; this was identified as DNA but the method used is not stated. Tovarnickij and co-workers isolated from their preparation 12 per cent. of nucleic acid which gave a positive Feulgen reaction, but no other properties of this nucleic acid are given. These analyses show that DNA is a constituent of rickettsiae, but the methods of isolation and analysis do not exclude the possible presence of RNA. For example, Tovarnickij and co-workers extracted the nucleic acid by the method used by Taylor and Sharp (1943), which consists of treatment with 5 per cent sodium hydroxide at 0–5° for 2 hr. and is sufficient to bring about appreciable hydrolysis of RNA to nucleotides.

With the aid of chromatographic techniques we have examined quantitatively the nucleic acid composition of *Rickettsia burneti*. This rickettsia was first investigated in Australia by Burnet and Freeman (1937), and independently in the United States by Davis and Cox (1938). It causes a pneumonia in man known as Q fever, but also infects cattle, sheep and goats. *R. burneti* resembles other pathogenic rickettsiae in its morphology, staining reactions, behaviour in experimental animals, and ability to multiply in an arthropod host (the tick). It differs from other pathogenic rickettsiae, however, in its ability to pass through filters with an average pore diameter as small as 400 mμ. Microscopically it is pleomorphic, but is frequently seen as a short rod 200–300 mμ. in diameter and 600–800 mμ. long. It is also more resistant than other rickettsiae to certain chemical and physical agents, and Philip (1948) has suggested that all these differences merit a separate genus *Coxiella* within the family *Rickettsiaceae*. *R. burneti* grows very profusely in the yolk sac of the fertile hen's egg, from which purified rickettsial suspensions may be prepared. In addition it was possible to isolate and analyse the DNA from the host for direct comparison with that of the rickettsia.

MATERIALS AND METHODS.

Preparation of rickettsial suspensions.

The procedure used for the preparation of purified rickettsial suspensions was based on Wolfe and Kornfeld's modification of Plotz's method (Wolfe and Kornfeld, 1949; Plotz, 1943). In this method non-rickettsial material is removed from crude formaldehyde-treated yolk-sac suspensions by centrifugation and treatment with ethyl ether.

Fertile hen's eggs were incubated at 38° for 6 days. The yolk sacs were then inoculated with a stock suspension of the Henzerling strain of *R. burneti* diluted in 10 per cent horse serum broth. This strain had already been adapted to growth in eggs. After incubation for 5–6 days at 35°, when about one-third of the embryos had died, the yolk sacs from all the eggs were harvested and stored

at — 25°. When smears showed bacterial contamination or only small numbers of rickettsiae the yolk sacs were discarded.

When 300–400 g. of infected yolk sacs had accumulated these were thawed, weighed, rapidly homogenized in a mechanical blender and made up to 20 per cent w/v in a diluent with the following composition: 0·85 per cent NaCl in distilled water, 4 parts; Sörenson's buffer pH 7·0, 1 part; the whole solution containing 0·5 per cent formalin. After standing at 2° for 48 hr. the suspension was centrifuged at 3600 r.p.m. (approx. 4500 g.) for 1 hr. (or at 11,000 r.p.m. for 30 min.). The pellet was re-suspended in the original volume of diluent and vigorously shaken for 1 min. with an equal volume of ethyl ether. After separation overnight in the cold room, the ether layer and the interphase, where most of the cellular debris had accumulated, were discarded. The aqueous phase, containing most of the rickettsiae, was centrifuged again as before. The pellet was then re-suspended in 1/5 the original volume of diluent, the suspension shaken with half its volume of ether, and left at 2° overnight. The aqueous phase was then repeatedly shaken with 1/4 vol. of ether and allowed to separate at room temperature for about 1 hr. After 3 or 4 ether treatments no interphase layer could be seen. The rickettsiae in the aqueous phase were then sedimented by centrifuging for 30 min. at 12,000 r.p.m. in a Sorval S.S.1 angle centrifuge, and washed by centrifugation 3 times in the buffered saline containing formalin.

From examination of stained smears the final preparations obtained by this method appear to consist entirely of rickettsiae, and this has also been confirmed by electron microscopy. The repeated washing by centrifugation would remove all extraneous particles of low molecular weight, and any material that might be loosely adsorbed on the surface of the organisms. However, there remained the possibility that the preparation might contain host particles of rickettsial size, indistinguishable microscopically from the organisms. In view of this, yolk sacs inoculated with 10 per cent horse-serum broth alone were submitted to the purification procedure described above. The final deposit, which was not visible to the naked eye, was made up to a volume equivalent in terms of starting material to that of the rickettsial suspensions, and the ultra-violet absorption spectrum of each examined in the Beckman photoelectric spectrophotometer. The absorption curve of the rickettsial suspension (Fig. 1) shows a peak at 255 mμ. due largely to the nucleic acid content, while the material isolated from normal eggs has only a small peak at 280 mμ., presumably due to aromatic amino-acids contained in protein, and is less than 1 per cent that of the rickettsial suspensions.

Analysis of the nucleic acids.

The isolation of purified nucleic acids from rickettsiae would not have been possible without the use of larger quantities of the organisms than could conveniently be prepared. However, the information required, namely the proportions of the component nucleotides in the nucleic acids, could be obtained by a simple direct method. This consisted of the extraction of the total nucleic acid components, the hydrolysis of these to the pyrimidine and purine bases, and their chromatographic separation and estimation, together with a similar extraction and analysis of the DNA after removal of RNA by hydrolysis with alkali.

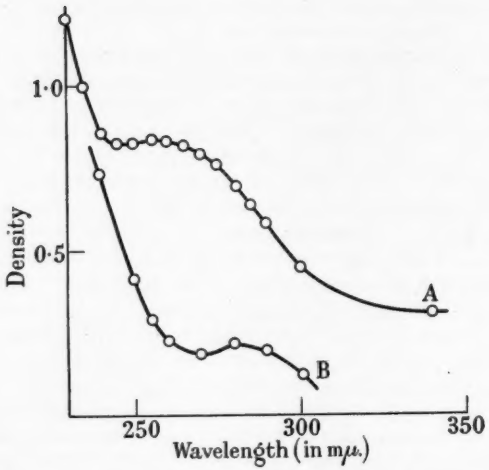

FIG. 1.—Ultra-violet absorption spectra of rickettsial suspension containing 88 μg./ml. (curve A), and material obtained by the purification procedure from normal yolk sacs (curve B). In terms of starting material A is at a dilution 20 times that of B.

Total nucleic acids.—By three successive treatments with 5 per cent trichloracetic acid at 90° the total nucleic acid components were extracted from 5–10 mg. of rickettsiae. The extract was evaporated to dryness and hydrolysed in 72 per cent w/w perchloric acid at 100° to liberate the purines and pyrimidines (Marshak and Vogel, 1950). Marshak and Vogel give the duration of the hydrolysis as 1 hr., but we have found that in the presence of small amounts of protein it is necessary to extend this to 2 hr. in order to ensure complete hydrolysis of the pyrimidine nucleotides from RNA. The increase in the hydrolysis time is not accompanied by any destruction of the purine and pyrimidine bases. The hydrolysate was diluted with an equal volume of water, centrifuged (to remove carbon derived from the sugars), and aliquots placed on a paper chromatogram which was run with 65 per cent *iso*propanol in water, 2N with respect to HCl (Wyatt, 1951*b*). This solvent resolves the purines and pyrimidines which were detected on the paper by the photographic method of Markham and Smith (1949), eluted in 0·1N HCl and estimated spectrophotometrically.

Deoxypentose nucleic acid.

Wyatt (1951*b*) has shown that treatment with N NaOH at 37° does not alter the quantitative composition of DNA. This was therefore used for the removal of RNA as nucleotides, preliminary to obtaining the quantitative composition of the rickettsial DNA (Schmidt and Thannhauser, 1945). The rickettsiae (8–18 mg.) were incubated with N NaOH at 37° for 12 hr. Addition of glacial acetic acid to pH 4 and one volume of ethanol precipitated the DNA together with some protein, leaving the ribonucleotides from RNA in solution. The precipitate was collected by centrifugation, washed with 50 per cent ethanol at

pH 4, extracted with hot trichloroacetic acid, hydrolysed and chromatographed as described above.

For the estimation of 5-methyl cytosine in DNA we used the technique described by Wyatt (1951*a*).

Isolation of DNA from normal chick embryos.

Mirsky and Pollister (1946) have isolated deoxyribonucleoproteins from several animal tissues by the removal of the bulk of the cytoplasmic components including the ribonucleoproteins by extraction with 0·14M NaCl, followed by extraction of the deoxyribonucleoproteins with M NaCl. This method was not very satisfactory for the isolation of DNA in the deoxyribonucleoproteins from chick embryos, apparently because of dissociation of the nucleoproteins.

The following method was found more useful: Normal chick embryos harvested after 12 days' incubation were mixed with ethanol in a mechanical blender (2 ml. ethanol/g. fresh wt.). The ground tissue was collected by centrifugation and extracted again with ethanol and with ethyl ether until most of the fats had been removed. It was then dried at 50° and incubated in a small volume of N NaOH at 37° overnight. The solution was centrifuged to remove material insoluble in the alkali, and the supernatant brought to pH 4 with acetic acid and one volume of ethanol added. After standing, the precipitate containing the DNA was collected by centrifugation and extracted with water brought to pH 8 with sodium bicarbonate. Protein was removed from the extract by gel formation on shaking with a chloroform-octanol mixture (chloroform 8 parts, octanol 1 part, v/v; Sevag, Lackman and Smolens, 1938). The nucleic acid was precipitated with ethanol and acetic acid and dried at 110°.

Nitrogen.

Nitrogen was estimated by a micro-Kjeldahl method.

Phosphorus.

Phosphorus was determined by the colorimetric method of Allen (1940).

Dry weight.

All dry weights were determined after drying at 110°.

Qualitative amino acid analysis.

The rickettsiae (1·76 mg.) were hydrolysed in 6N HCl for 6 hr. at 120°. After removal of HCl by vacuum distillation the amino acids in the hydrolysate were separated by two-dimensional paper chromatography using butanol-acetic acid-water and phenol-water-ammonia as solvents. They were detected by the usual ninhydrin method.

RESULTS.

Composition of the rickettsial nucleic acids.

Three separate preparations of *R. burneti* were used in these analyses. A portion of the rickettsial suspension from preparation 1 was dialysed against distilled water and made up to a known volume for the determination of dry weight, nitrogen and phosphorus. The total yield from 344 g. of yolk sacs (wet

weight) was 44 mg. (dry weight). This contained 13·2 per cent N and 1·8 per cent P.

The purine and pyrimidine composition of the total nucleic acid fraction was estimated in two preparations (1 and 2), while the composition of the DNA from all three preparations was determined. Adenine, guanine, cytosine, uracil and thymine were identified on the chromatograms of the total nucleic acid bases. Both RNA and DNA thus appeared to be present, and this was confirmed by the complete removal of uracil together with a portion of the adenine, guanine and cytosine after hydrolysis with N NaOH at 37°. The compositions of the DNA from all three preparations were identical within the limits of experimental error. It was thus possible from the amount of thymine in the total nucleic acid fraction to calculate the quantities of adenine, guanine and cytosine accounted for as DNA, and hence, by subtraction, obtain the quantities of the bases present in the RNA. (It should be pointed out here that the adenine fraction of the RNA as estimated would include any adenine present as free adenylic acid or its derivatives.) Expressed on a dry weight basis the amounts of DNA in the two preparations are identical but the RNA content shows some variation (Table I).

TABLE I.—*The Nucleic Acid and Phosphorus Content (per cent dry weight) of Preparations of* Rickettsia burneti.

	Preparation 1.	Preparation 2.
DNA	9·8	9·5
RNA	2·95	5·5
P	1·81	..
P accounted for as nucleic acid	1·30	..

5-methyl cytosine could not be detected on the chromatograms of the DNA hydrolysates run in the *iso*propanol-HCl solvent, nor was this substance observed when the area corresponding to the position of this base was eluted and the concentrated eluent chromatographed using a second solvent. On elution and spectrophotometric examination, the area corresponding to 5-methyl cytosine on the second chromatogram showed no ultra-violet absorption characteristic of this substance. This enabled a definite limit to be set to the amount of 5-methyl cytosine that would have passed undetected. The compositions of DNA from the three preparations of rickettsiae are given in Table II.

TABLE II.—*Ratios of Purines and Pyrimidines in Deoxypentose Nucleic Acids from* Rickettsia burneti *and Chick Embryos.*

	R. burneti DNA.			Embryo DNA.			Significance of the difference between the two means (P.).
	Number of preparations analysed.	Mean molar values.*	S.E.	Number of preparations analysed.	Mean molar values.*	S.E.	
Adenine	3	**1·18**	±0·012	3	**1·17**	±0·018	0·5 –0·7
Guanine	3	**0·90**	±0·015	3	**0·96**	±0·012	0·02–0·05
Cytosine	3	**0·88**	±0·020	3	**0·82**	±0·004	0·02–0·05
Thymine	3	**1·04**	±0·010	3	**1·06**	±0·009	0·2 –0·3
5-methyl cytosine	2	**<0·008**		2	**0·037**		

* The molar values are calculated such that those for the 4 major bases (adenine, guanine, cytosine and thymine) total 4.

Deoxypentose nucleic acid from chick embryos.

During growth in the fertile egg the rickettsiae proliferate in the cells of the of the yolk sac. This would therefore have been the most suitable material for the preparation of host DNA for comparison with that of the rickettsiae. However, only small amounts of DNA could be isolated from yolk sacs, whereas appreciable yields were obtained from the whole embryo. DNA from different tissues of the same species have been found to show the same composition (Chargaff, 1950 ; Wyatt, 1951*b*). There is no reason, therefore, to suspect that the DNA of the yolk sac differs in composition from that isolated from the whole embryo.

Three preparations of chick embryo DNA were analysed, and in Table II the results are compared with those of the DNA from rickettsiae. The small differences in the proportions of adenine, guanine, cytosine and thymine between the two nucleic acids are on the borderline of significance. The nucleic acids differ, however, in their content of 5-methyl cytosine. While the embryo DNA contains a proportion of 5-methyl cytosine equal to 4·2 per cent of the cytosine, in the rickettsia DNA this base could not be detected, and must be less than 0·98 per cent of the cytosine.

The amino acids of R. burneti.

Qualitative paper chromatography showed the presence of alanine, arginine, aspartic acids, cystine, glutamic acid, glycine, histidine, leucines (probably an unresolved mixture of leucine and isoleucine), lysine, methionine, phenylalanine, proline, serine, valines, and a trace of threonine. Tyrosine could not be detected on the chromatogram, suggesting that it cannot be present in more than about 0·05 per cent by weight. It is probably present in small quantities as the rickettsiae give a faint reaction with Millon's reagent. Under the conditions used for the formaldehyde inactivation of the organisms only the free amino groups in the proteins would combine and this reaction would be incomplete. There is little reason to believe that the low tyrosine content is due to the formaldehyde treatment.

DISCUSSION.

The bacteria which have been examined contain both RNA and DNA, and this is generally believed to hold for all bacteria. The early investigations on the nucleic acids of viruses, largely based on colour reactions of the sugars, showed that plant viruses contained ribonucleic acid and animal viruses deoxypentose nucleic acid. It now appears that the viruses may be divided into three groups on the basis of their nucleic acids ; the plant viruses (at least those which can be purified), and possibly certain animal viruses, which contain only RNA ; the bacteriophages (Cohen and Arbogast, 1950) and the insect polyhedral viruses (Bergold, 1947) containing only DNA ; and some animal viruses such as influenza and vaccinia which possess both types of nucleic acid (Knight, 1947 ; Wyatt, unpublished). In containing both RNA and DNA *R. burneti* resembles the bacteria and the larger animal viruses.

The quantitative compositions of deoxypentose nucleic acids from some bacteria and viruses have been studied by Smith and Wyatt (1951). The range of compositions among microbial DNA is very much wider than that of DNA from higher animals. However, DNA from different strains of the same bacterium

or virus show very similar compositions. In contrast to that from animals and plants, the DNA from the micro-organisms studied contains no 5-methyl cytosine. This is also true of *R. burneti*, and possibly the absence of this pyrimidine is a characteristic of all microbial deoxypentose nucleic acids.

For reasons stated elsewhere in this paper we do not believe that our rickettsial preparations were contaminated to any measurable extent with host cell material. It is interesting then that in the proportions of the four major bases (adenine, guanine, cytosine and thymine) the rickettsia DNA is almost identical with the host DNA; the two nucleic acids differ only in that one contains 5-methyl cytosine and the other apparently does not. In the case of the bacteriophages T2 and T5 and their host *Bact. coli* the deoxypentose nucleic acids of host and virus differ strikingly in composition (Smith and Wyatt, 1951). There is no reason to suppose that the DNA present in any cell consists of a single molecular species, and the nucleic acid as analysed may be an aggregate of several different types of molecule with differing compositions. In the embryo nucleic acid, for instance, it is possible that only one or a small number of these contains the 5-methyl cytosine. It is not possible therefore to dismiss the possibility that host nucleic acid may be used directly in the proliferation of the rickettsiae.

It would, of course, be desirable to study the nucleic acid composition of this organism grown in a host with a nucleic acid composition different from that of the chick embryo. This might be possible by growth in the mouse lung, although for technical reasons it is less easy to isolate sufficient quantities of washed rickettsial suspensions from this source.

SUMMARY.

Suspensions of *R. burneti* have been prepared which are believed to contain little, if any, material derived from the host cell. The compositions of the nucleic acids present in the rickettsiae in these preparations have been examined quantitatively, using paper chromatography and spectrophotometric methods.

R. burneti contains 9·7 per cent (dry weight) deoxypentose nucleic acid and about 4·3 per cent ribonucleic acid. Different preparations have the same content of DNA but the amounts of RNA differ. The nucleic acids do not account for all the phosphorus in the organisms.

The purine and pyrimidine composition of the rickettsial DNA has been determined. No 5-methyl cytosine could be detected in this nucleic acid.

Deoxypentose nucleic acid has been isolated from normal chick embryos and its purine and pyrimidine composition determined. The proportions of adenine, guanine, cytosine and thymine in this nucleic acid do not differ appreciably from that of the rickettsial DNA. The embryo nucleic acid, however, contains 5-methyl cytosine amounting to 4·3 per cent. of the cytosine content.

The amino-acids present in the rickettsiae have been identified by paper chromatography.

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THE ACTION OF AMMONIA ON COMPLEMENT IN THE INACTIVE PHASE ASSOCIATED WITH HIGH CONCENTRATIONS OF SODIUM CHLORIDE.

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THE inactivation of complement by salt action has been studied by many workers. One of the most interesting experiments in this field was that of Muir and Browning (1909), who showed that complement could be inactivated by a hypertonic concentration of sodium chloride, that in such a state it could pass freely through a Berkefeld candle, and that its activity could be restored after filtration by diluting to the level of 0·85 per cent. sodium chloride. Untreated active complement, however, was largely retained by the filter. Wright and MacCallum (1922) made a wide study of the inhibition of complement activity by salts. Following up this work Gordon and Thompson (1933*a*) made a study of the action of neutral non-hydrolysable salts of sodium and potassium. The results gave the lyotrope series for the salt effects found with gelatin, and it was argued that a dispersion of the serum proteins was the most probable cause of the inhibition observed, and that complement activity was necessarily associated with a particular state of aggregation of the serum proteins. In a latter paper Gordon and Thompson (1933*b*) showed that the effect of sodium and potassium chlorides, nitrates and bromides appeared to be completely reversible on dilution even after twenty-four hours' action of high concentrations.

In their early work on complement, Gordon, Whitehead and Wormall (1926) described a component which was inactivated by small quantities of ammonia. It was relatively heat-stable, not being destroyed by heating at 55° for half an hour and was associated with the "albumen" fraction, i.e., the protein not precipitated from diluted serum by CO_2. The activity of the complement could be restored by the addition of small amounts of heated serum. Because of three other components that had been described, the authors suggested that it be known as the fourth component. Misawa in 1932 had found that this component, while in hypertonic NaCl, could be destroyed by the action of ammonia as completely as when it was in isotonic solution. This observation acquired an added significance in the light of the later findings of Gordon and Thompson (1933*a* and *b*) referred to above, hence it was thought desirable to confirm it with more precise experiments than those of Misawa, who did not give complete experimental details.

EXPERIMENTAL.

Six ml. of a 10 per cent solution of sodium chloride was added to 8 ml. guinea-pig serum in distilled water. This mixture was allowed to stand at room temperature for two hours. On adding quantities of 0·1, 0·2, 0·3, 0·4 and 0·5 ml. of this mixture to sensitized red blood cells, haemolysis occurred only when the dilution was highest. To two 6 ml. amounts of this serum-salt mixture were added 0·2

and 0·3 ml. of N/1 ammonia; they were then incubated for 2½ hr. at 37° and neutralized with N/1 acid. On adding 0·1 ml. of each of these mixtures to sensitized red blood cells and incubating at 37° for 1 hr. there was no haemolysis, although the dilution was such that if the complement had been inactivated by sodium chloride alone it would have been reversed by the dilution. The addition of 0·1 ml. heated serum to such inactivated serum restored its complement activity. The inference suggested is that the inactivation of the complement by the addition of sodium chloride with the dispersion of the serum proteins does not interfere with the action of ammonia on the proteins. The results are given in the Table.

SUMMARY AND CONCLUSIONS.

These observations suggest that the action of the sodium chloride is by physical change affecting only the degree of dispersion of the proteins, and that the molecule has undergone no irreversible chemical change.

Ammonia can still inactivate complement in this dispersed phase, so that on diluting it to isotonicity no activity is found although it can be restored by adding heated serum.

I should like to express my indebtedness to the Medical Research Council for an expenses grant.

TABLE—*The Effect of a Strong Solution of Sodium Chloride on Complement and its Restoration on Dilution*

x = 8 ml. guinea-pig serum + 6 ml. 10 per cent sodium chloride solution.

				Degree of haemolysis.
1	0·1 ml. of x +	0·4 c.c. of 5 per cent sensitized ox red cells	+ 0·2 c.c. normal saline	4
2	0·2 ml. of x +	Ditto	+ „ „ „	3
3	0·3 ml. of x +	„	+ „ „ „	0
4	0·4 ml. of x +	„	+ „ „ „	0
5	0·5 ml. of x +	„	+ „ „ „	0

The Effect of Ammonia on Serum containing a high Concentration of Sodium Chloride

12 ml. of x { (A) 6 ml. + 0·2 ml. N/I ammonia } incubated for 2½ hours and neutralized
{ (B) 6 ml. + 0·3 ml. N/I ammonia } with N/I HCl at 37°.

				Degree of haemolysis.
6	0·1 ml. of (A) +	0·4 ml. of 5 per cent sensitized ox red cells	+ 0·2 ml. normal saline	–
7	0·1 ml. of (B) +	Ditto	+ „ „ „	–
8	0·1 ml. of (A) +	„	+ 0·1 ml. „ „ „	
			+ 0·1 ml. serum heated at 55° for ½ hour	4
9	0·1 ml. of (B) +	„	+ 0·1 ml. normal saline	
			+ 0·1 ml. serum heated at 55° for ½ hour	4
10	0·1 ml. heated serum +	0·4 ml. of 5 per cent sensitized ox red cells		0

4 = Complete haemolysis. 3 = Almost complete haemolysis. 0 = No haemolysis.

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A TECHNIQUE FOR TOTAL HEPATECTOMY IN THE RAT AND ITS EFFECT ON TOXICITY OF OCTAMETHYL PYROPHOSPHORAMIDE.

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OCTAMETHYL pyrophosphoramide (OMPA) is the active principle in a number of insecticides that are being used on an increasingly wide scale. An account of some of its toxic properties has been given by Dubois, Doull and Coon (1950). It has also been used successfully for the treatment of myasthenia gravis (Rider, Schulman, Richter, Moeller and Dubois, 1951).

When a toxic dose of OMPA is administered to an animal the signs of poisoning by a cholinergic drug make their appearance within 15–30 minutes and the level of cholinesterase in the blood and some of the tissues falls. For rats, an average lethal dose of OMPA is 8 mg. per kg., and is the same if the drug is administered orally or by parenteral injection.

On the other hand, OMPA, unlike other cholinergic poisons, has no inhibitory action on blood or tissue cholinesterase *in vitro*. However, when liver slices or liver mince is incubated with OMPA an active inhibitor of cholinesterase is produced. No other tissues examined could be shown to possess this power of converting OMPA *in vitro* to an active inhibitor of cholinesterase (Dubois *et al.*, 1950 ; Gardner and Kilby, 1950 ; Aldridge, 1951).

It was, therefore, of interest to find out whether the conversion of OMPA into a cholinesterase inhibitor takes place in the liver of an intact animal.

For this purpose we devised a method by which the rat, a convenient small laboratory animal, could be rendered liverless. The main problem to overcome was that of establishing an adequate collateral circulation for the portal vein and the inferior vena cava before the liver is removed. After considerable trial this difficulty was overcome and a successful 2-stage technique for total hepatectomy in rats was developed.

The hepatectomized rats survived as long as 10 hours without any treatment. The technique will be described in some detail, because it may be of value to those wishing to use rats for metabolic experiments. While this paper was being prepared a brief reference to a similar technique in rats was found (Byers, Friedman and Michaelis, 1951), but no operative details were given.

The effect of injecting OMPA into the hepatectomized rats was observed. The animals survived the administration of doses that killed control animals in 12–23 minutes.

TOTAL HEPATECTOMY

First stage.

Large rats weighing over 300 g. are used. Under ether anaesthesia and observing simple aseptic precautions a right paramedian incision 6 cm. long is

made from the costal margin. After placing a pad of cotton-wool 1–2 cm. thick under the animal's spine at the level of the last rib, intestine is delivered outside the wound, wrapped in a moist towel and drawn to the left. This exposes the right lobe of the liver and the inferior vena cava. The ligament connecting the inferior pole of the right posterior lobe of the liver to the inferior vena cava is divided and a segment of inferior vena cava above the entrance of right renal vein is dissected free in its bed of tissue. Polythene ("alkathene," gauge 300, I.C.I. product) and cellophane films of roughly 15 mm. square are sterilized by boiling in water and placed together. The two wet films adhere closely together and can be treated as a single sheet. A band of about 3 mm. in width is cut from this and passed round the isolated segment of the inferior vena cava with cellophane as the inner layer. After placing a tapering glass rod longitudinally on the vein, the two ends of the constricting band are tied together with a single stitch of fine steel wire so as to include both the rod and the vein. When the glass rod is removed, the inferior vena cava is left half occluded by the band. Further vascular constriction is produced by the fibrosis resulting from the presence of the cellophane. The redundant ends of the band are cut off. The median and left lateral lobes of the liver are gently retracted upwards and the portal vein is exposed. Near its entrance into the liver it is dissected free from adjacent soft tissue, the hepatic artery and the common bile duct. A constricting band is also placed round it to occlude half of its lumen. The incision is closed in two layers with fine steel wire. No special post-operative care is required. At least 6 weeks should be allowed for the development of adequate collateral circulation.

Second stage.

The rat is deprived of food for 16 hours. Under ether anaesthesia the abdomen is opened in the midline starting on the xiphisternum and continuing for about 7 cm. posteriorly. The dilated abdominal veins should not be injured. A pad of cotton-wool is placed under the spine to raise the upper part of the abdominal wall. The median and the left lateral lobes of the liver are expressed outside the incision from the cupola of the diaphragm by squeezing the lateral sides of the upper abdominal wall with the fingers. After dividing the falciform ligament down to the vena cava, the left coronary ligament is exposed by drawing down the left lateral lobe of the liver and divided. The two lobes are cut off after their pedicle has been securely ligated with linen thread. The gastro-hepatic omentum is split longitudinally in the middle and the anterior portion of the caudate lobe is delivered outside the opening. The posterior portion of the caudate lobe, which is situated behind the oesophagus, is also brought outside through the same opening after dividing the overlying peritoneal fold which is partly attached to its ventral surface. The medial side of the remaining liver is now free. Turning to its lateral side, the right coronary ligament is cut after exposing it by holding the right lobe of the liver and pulling it downwards. The right lobe is lifted from the posterior abdominal wall to expose the peritoneal fold between the liver and the crus of the diaphragm. It is divided, and two linen threads are passed from the lateral side of the liver through the opening to its medial side. The two ends of one thread are tied in a loose half hitch round the inferior vena cava between liver and diaphragm.

The first operation invariably causes formation of adhesions between the liver, duodenum and omentum and around the constricting bands. However, if the operation for the first stage has been skilfully done with a minimum of trauma the adhesions are not great, enabling these parts to be separated by rubbing off the liver with gauze and the fingers while controlling the oozing of blood during their separation by compression. When this is done, the two ends of the second thread are brought to the front under the liver and tied tightly. This ligature embraces the portal vein, the bile duct and the inferior vena cava below the liver. The first ligature is then tightened and the intervening liver is cut off as close to the ligature as possible. After removing the spilled blood from the peritoneal cavity, a gauze sponge is left in the operated site to facilitate haemostasis. The abdominal wound is closed with two layers of linen sutures. The operation lasts about 15 minutes. However, if adhesions are extensive much time is consumed in separating them, with great loss of blood. These animals are in very poor general condition after the operation and die soon afterwards. Thus such animals are best discarded.

Clinical Course.

On recovering from the anaesthetic the rat sits up and remains quiet most of the time, but occasionally moves about. It appears anxious and irritable. Later, marked weakness is apparent with sprawling limbs and head resting on the floor. When moribund it lies on its side. The respiration also becomes deeper and slower and Cheyne-Stokes breathing appears in the terminal stage. Fine fibrillary muscular tremors, starting from the head and becoming generalized later, precede the convulsions, which are usually associated with marked opisthotonos. Occasionally the animal rolls on its side. It dies after 2 or 3 such convulsions with respiration stopping before the heart beat.

In our series the hepatectomized rats lived 3–10 hours without administration of glucose. Their period of survival after operation depends on technical skill, extent of operative trauma, amount of blood loss, adequacy of collateral circulation and on keeping the rat quiet without disturbance.

Effect of OMPA on Hepatectomized Rats.

A 1 per cent. aqueous solution of OMPA in a dose of 30 mg. per kg. body-weight was injected into the external jugular vein of male rats immediately after total hepatectomy and after sham operation. A third group of rats was only given an intravenous injection of 0·9 per cent. saline after total hepatectomy.

The results show that liverless rats are able to survive a dose of OMPA sufficient to kill sham-operated rats in 12–23 minutes (Table 1). The mean survival time

TABLE I.—*Period of Survival of Hepatectomized and Sham-Operated Rats after Intravenous Injections of OMPA* (30 *mg. per kg. body-weight*).

Group.	No. of rats	Period of survival. Mean ± S.E.	Range.
Sham-operated, OMPA injected.	12	17·50 ± 0·89 mins.	12–23 mins.
Hepatectomized :			
a. OMPA injected.	11	5·25 ± 0·33 hrs.	3·2–7·1 hrs.
b. Saline injected.	11	6·05 ± 0·78 hrs.	3·0–10·9 hrs.

of the hepatectomized rats given OMPA was not significantly shorter than that of hepatectomized rats injected with saline only. Both groups of rats died with hypoglycaemic convulsions, but those given OMPA showed some signs of poisoning, namely lachrymation, salivation and muscular twitchings, which developed 4–5 hours after injection. This suggests that some tissue or tissues other than the liver could bring about a slow conversion of OMPA to an active anticholinesterase agent.

DISCUSSION.

These experiments have shown that it is possible to perform total hepatectomy on rats, their survival periods being up to 10 hours. Earlier experiments showed that success depends to a great extent upon the development of an adequate collateral circulation, and a minimum period of 6 weeks seems to be necessary for this. Such rats prove to be just as sensitive to the action of an active anticholinesterase drug, e.g., *p*-nitrophenyl diethyl phosphate (E. 600), as normal or sham-operated animals.

Nothing is known about the chemical reactions involved in the conversions of OMPA into an active cholinesterase inhibitor, nor has the inhibitor been identified or even suggested. The present experiments indicate that the change only takes place at an effective rate in the liver, and they confirm the results of *in vitro* experiments.

SUMMARY.

1. A 2-stage technique for total hepatectomy in rats is described.

2. Hepatectomized rats survived the injection of a dose of OMPA which killed sham-operated rats.

I am grateful to Dr. J. M. Barnes and Mr. B. B. Milstein for helpful suggestions, and to Miss Jean Bennett for technical assistance.

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THE DEVELOPMENT OF THYMIC BODIES IN RATS AFTER FAT INJECTION.

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THE purpose of this paper is to draw attention to the development of peculiar cystic structures in the thymus of the rat, apparently as a result of various different stimuli. These structures have been described before, principally by Ross and Korenchevsky (1941) after injection of oestrogens into gonadectomized rats, and are known to occur in a less developed form occasionally in normal mammals and especially in dogs (Cowdry, 1928). However, little attention appears to have been paid in the literature to these structures and their significance.

During the course of some experiments for another purpose, involving the injection into rats of various extracts of fats over a period of some weeks, it was discovered that these structures were developing fairly frequently, irrespective of the fat used ; accordingly their development under conditions not previously reported is felt worthy of recording.

MATERIALS AND METHODS.

Three-months-old male and female Wistar strain rats were used, fed on compressed meal pellets, green-stuffs and water *ad lib*. Two separate series of experiments were conducted, involving the daily intraperitoneal injection of 0·5 ml. of ethereal extract of fat (human mesenteric, calf mesenteric or calf thymus). The extracts were prepared as follows : The material was frozen and then minced and placed in a large flask fitted with a mechanical stirrer. The flask was then three-quarters filled with anaesthetic ether and the mixture heated to boiling point under a reflux condenser for four hours. The ether was then poured off and a fresh quantity introduced and the process repeated twice for periods of two hours each. The combined extracts were reduced by boiling off on a steam bath until a thick semi-liquid material and some water was left. Some fresh ether was added to this residue, solution being aided by a little very gentle heating, allowed to cool and the supernatant liquid decanted off (the water being left behind) and filtered. The supernatant liquid was again evaporated down and the process repeated. The final product was a thick yellowish substance tending to solidify at room temperature. Before injection it was warmed slightly, and could then be drawn easily into the syringe.

The first series of experiments involved 56 male and female rats which were kept segregated. Six were used as untreated controls. The rest were divided into two equal groups, one receiving calf thymus fat extract, the other human fat extract, for a total of 56 days. Three animals died before the end of the

first 3 days and were discarded. The remainder were killed in batches of 4 or 5 at weekly or fortnightly intervals.

The second series used 52 male and female rats. Four were untreated controls, and the remainder were divided into 3 equal batches and injected as before with 0·5 ml. of human fat extract, calf thymus fat extract, or calf fat extract. These rats were killed in batches of 4 from each group at weekly intervals for 4 weeks.

All the animals were killed 1 hour after that day's injection by stunning and cutting of the throat, the blood being collected for chemical analysis. The organs were removed and fixed in Bouin's fluid or 10 per cent formalin for histological examination, the thymus unless small being cut into two portions.

RESULTS.

TABLE—*Numbers of Rats Developing Thymic Bodies.*

No. of animals.	Material injected.	Animals developing thymic bodies.		
		Definite.	Suggestive.	Possible.
39	Thymus fat	12	2	2
40	Human fat	8	2	—
16	Calf fat	6	1	3
10	Untreated	1	—	—

The Table shows the totals from the two series of experiments of animals developing these peculiar cystic structures hereafter called "thymic bodies." During the course of the experiments the thymus at first involuted, and in the involuted state there was never any sign of these structures. In animals killed after the first 14–21 days the thymus was found to be regenerating, and it was in this stage or later that the thymic bodies occurred. No special correlation between the number of thymic bodies and duration of treatment was found.

In the 10 untreated controls one thymus which was abnormal in that it showed a cystic degeneration also had a small thymic body. Another thymus had a small circular cyst, consisting of a single layer of ciliated columnar epithelium with a lumen containing vacuolated acidophile material; a small blood vessel was present running along the lower end of the cyst, which was situated deep in the medulla of the thymus and not related to any fibrous septum, unlike a true thymic body, and accordingly it was not included even as a possible thymic body. In no other untreated control was there any suggestion of a thymic body.

The true thymic bodies were situated alongside, or at the end of, the thin fibrous septa which separate the lobules of the thymus, and though often occurring in single groups, as many as four groups have been found in one section, and two were commonly found.

With haematoxylin and eosin staining, these structures consisted of a mass of somewhat basophile-staining cells frequently having an acinar arrangement (see Fig. 1–4). The cells lining the acini were cubical or low columnar in shape but no ciliated epithelium was found. The cells not lining the acini had a profuse and slightly basophile cytoplasm with a round nucleus showing a chromatin network but no nucleolus, and an indefinite but usually polygonal shape.

The lumina of the acini were empty, or filled with a granular acidophile material with some nuclear debris or occasionally some cells resembling lympho-

cytes. In serial sections the acinar spaces in each body appeared to be continuous, suggesting that the structure was of the nature of a coiled and very tortuous tube.

Frequently a thin capillary blood vessel ran in close juxtaposition to these cell groups, but there did not appear to be any connection between the acinar spaces and the blood vessel. Around the cell masses a very thin fibrous strand (one cell thick) was occasionally seen, but this was not a constant feature.

These bodies stand out prominently and were easily detected if present. In serial sections the bodies appeared and disappeared very suddenly, and therefore these structures might have been easily missed unless numerous sections had been taken.

DISCUSSION.

The origin and significance of these bodies is obscure. Hassall's corpuscles are known to be scanty and very poorly developed in the thymus of the rat, and in our experiments the few Hassall's corpuscles present were neither larger nor more numerous than in the normal rat ; it is hence unlikely that these bodies arise from them.

With regard to the incidence of thymic bodies in untreated controls, we can confirm the findings of Ross and Korenchevsky (1941) that these bodies were rare or very poorly developed. Plagge (1946), however, found small epithelial cysts, similar to the larger ones of the test animals, in six thymuses which had been studied in serial section out of a total of eleven control rats : although some of his controls had been injected with sesame oil, he states that " pure sesame oil had no influence on the occurrence of the structures " ; unfortunately he did not state the number of controls so treated.

Ross and Korenchevsky found that the thymic bodies occurred in gonadectomized rats after treatment with oestrogens or simultaneous injection of male and female hormones, but not after treatment with male hormones alone. Plagge (1946) found they occurred in intact rats after injection with oestrogen for long periods (4–10 months), and in his series they reached a considerable size with marked cyst formation. He considered that the size and number of cysts were approximately proportional to the length of treatment, but we have not found this.

Ross and Korenchevsky (confirmed by Plagge) ascribed their occurrence to a specific effect of the oestrogens, since hyperplasia of epithelial structures after oestrogen injections has been reported before in the uterus (Korenchevsky and Hall, 1940), seminal vesicles (Korenchevsky, 1941), and in the urinary bladder (Hume, Burbank and Korenchevsky, 1939).

EXPLANATION OF PLATE.

FIG. 1.—Thymus of rat injected with thymus fat. One of two thymic bodies with well-developed acinar spaces, most of which are filled with acidophile material and nuclear debris ; one acinus is empty. H. & E. × 100.

FIG. 2.—Thymus of rat injected with human fat. Thymic body with well-formed acini containing nucleated cells and debris. H. & E. × 150.

FIG. 3.—Thymus of rat injected with human fat. Straggling type of thymic body with poorly formed acini. H. & E. × 100.

FIG. 4.—Thymus of rat injected with calf fat. Thymic body running alongside a septum, with distinct acini containing granular material in upper portion. H. & E. × 150.

Davidson.

Their occurrence in our series of experiments in intact animals after injection of fat extracts with no apparent association with the sex hormones suggests that, while their size may be exaggerated by the oestrogens, their occurrence is a non-specific response of a similar type to those which occur in the adaptation syndrome (Selye, 1946).

The fact that the frequency of occurrence with each type of fatty extract was approximately equal tends to support the view that it is a non-specific response, but the fact that Ross and Korenchevsky did not find them after treatment with male hormones leads one to consider that another factor, at present obscure, may be operating as well.

In our series of experiments no epithelial hyperplasia was found in any other organ, including the sex organs, which suggests that there was no inherent oestrogenic effect in the fat extracts.

It would appear on balance that this must be a non-specific response to repeated stimuli, and the nature of these bodies is at present conjectural. Since they are closely associated, but not connected, with thin-walled blood-vessels, their function may be that of excretion of some metabolic product from the thymus, which is still being subjected to stimulation after recovery from its involution phase.

SUMMARY.

The occurrence of peculiar cystic epithelial structures called " thymic bodies " in the thymus after daily injection of extracts of calf thymus, calf fat and human fat into intact rats is described.

They appear similar to those found by other workers in gonadectomized and intact rats after treatment with certain hormones and thought to be a specific response to these hormones. From the present experiments it appears that they are non-specific in nature and not necessarily related to any hormone.

Their significance and function are obscure, but it is suggested that they may have an excretory function while the animal is still being treated ; they do not occur when the thymus is involuted, nor do they occur commonly in untreated normal rats.

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THE BEHAVIOUR OF INTRAVENOUSLY INJECTED PARTICLES OF CARBON AND MICROCOCCIN IN NORMAL AND TUBERCULOUS TISSUE.

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OBSERVATIONS previously reported (Markham, Wells, Heatley and Florey, 1951) showed that, although micrococcin inhibited the growth of tubercle bacilli *in vitro*, it only had a slight suppressive effect on the early development of tubercles when it was injected intravenously into infected rabbits and guinea-pigs. Although it is well known that fine particles injected into the blood stream are soon taken up by reticulo-endothelial cells such as exist in the liver and spleen, there appears to be no precise knowledge of what happens to the particles immediately after injection. In an endeavour to follow more exactly the march of events after the injection of finely particulate matter that is not agglutinated *in vitro* by serum, microscopic observations were made on the vascular system in normal tissue in transparent chambers inserted in rabbits' ears. Observations on tuberculous tissue were made by the same technique in order to gain more precise information on the distribution of intravenously injected micrococcin in such tissue.

EXPERIMENTAL.

Normal Tissue.

Chambers made from perspex were inserted into rabbits' ears by a technique similar to that described by Ebert, Florey and Pullinger (1939). The tissue which grew over the transparent table of the chamber was from 30 to 40μ thick, so that events in the blood vessels could be clearly followed microscopically.

Injection of a suspension of carbon.

A carbon suspension consisting of one part of Indian ink (Edward Gurr) diluted with three parts of distilled water was filtered once through a double thickness of filter-paper and boiled. This suspension consisted of particles which were not greater than 3μ in diameter, and usually considerably less. It was not agglutinated *in vitro* by horse or rabbit serum.

In the first experiment 10 ml. of the suspension were injected slowly into the marginal vein of the unoperated ear, while observations were made simultaneously on the circulation in the chamber in the other ear. About 5 minutes after the beginning of the injection emboli were seen passing along the vessels. The emboli, consisting of structureless material in which carbon particles were embedded, were of various sizes, some being large enough temporarily to block fine vessels. The larger masses were forced slowly through the vessels, and at

the end of 30 minutes no carbon, either intra- or extravascular, was observed in the chamber.

A similar observation was made in another rabbit using 8·0 ml. of the suspension. Agglutinated masses of about 10μ diameter were seen during the injection, which took 1½ minutes. None were seen after 4 minutes.

These preliminary observations with carbon showed that agglutination of particles occurred in the blood stream, although serum was without effect *in vitro*.

Injection of a suspension of micrococcin.

Very fine suspensions of micrococcin in 30 per cent rabbit serum diluted with 0·9 per cent NaCl were prepared as previously described (Markham, Heatley, Sanders and Florey, 1951).

In the first experiment 166·8 mg. of micrococcin in 10 ml. of diluted serum were injected into the unoperated ear. About 1½ minutes after the beginning of the injection fine particles were seen passing through the blood vessels in the chamber, but after 20 to 25 minutes particles were no longer recognizable. A few minutes after the particles were first observed in the blood stream some, recognizable as black dots, were seen adhering to the vessel walls. The impression was gained that micrococcin adhered best to endothelium at places where leucocytes tended to stick, though there were actually few such places at this time. Another experiment was accordingly done in which slight inflammation with sticking of leucocytes was caused by pressure on the mica cover of the chamber. After the injection of 200 mg. of micrococcin in 10 ml. of diluted serum, material was seen to adhere to parts of the vessel walls (Fig. 1). Probably this material was composed of micrococcin and platelets. It was noted that platelets, which are readily seen in the blood vessels in ear chambers, temporarily disappeared almost completely following the injection. Some white and red cells were also seen entangled in the masses.

Further observations were made on chambers in which the table area was not yet completely vascularized, using ultra-violet light, which makes micrococcin fluoresce brilliantly (Markham, Heatley, Sanders and Florey, 1951). The vessels in the growing edges of the tissue in such chambers are delicate and "sticky," and leucocytes adhere to them in considerable numbers.

In one experiment about 200 mg. of micrococcin in 9 ml. of diluted serum were injected intravenously in 5¾ minutes. Within about 8 minutes emboli of various sizes were seen by ordinary light to pass along the vessels. When, 15 minutes after the beginning of the injection, ultra-violet light was used, fluorescent particles were seen adhering to the walls of the vessels. It was difficult at the time to be certain that some particles were not extravascular, but from subsequent observations this was very unlikely. Some 4 hours later an injection of 5 ml. of the same suspension was given, and ultra-violet light was used for observation throughout. Within 2 minutes of the beginning of the injection brilliantly fluorescent masses were seen moving swiftly along the vessels. No small particles were recognizable. Some of the masses were 60 to 90μ long and about the width of the vessel accommodating them. Within 5 minutes plaques of fluorescent material, which had a yellowish tinge rather than the blue of pure micrococcin, adhered to the vessel walls, particularly in the growing region (Fig. 2). This fluorescent material remained in the vessels for several days in gradually diminishing quantity, none being present 10 days after the injections.

Similar observations were made with another animal. A suspension containing 202 mg. of micrococcin in 10 ml. was injected intravenously in 1 minute. Plaques of fluorescent material on the walls of the vessels, similar to those seen in the first animal, remained for several days, and some appeared to become extravascular because of the remoulding of the vascular pattern. Ten days after the first injection 500 units of heparin were injected followed by 200 mg. of micrococcin in 10 ml. of diluted serum. In spite of the heparin large plaques formed in the vessels.

Many fluorescent particles 1μ or less in diameter were seen in smears of blood taken soon after an injection. These particles dissolved in alcohol which, when evaporated on filter-paper, left a patch showing the typical blue fluorescence of micrococcin in ultra-violet light.

That micrococcin was sometimes transferred from the vessels to tissue macrophages was shown in one animal. Nine days after an injection the vascular pattern at the growing edge had greatly changed, and some micrococcin, which had probably been within vessels now eliminated, was seen in the extravascular macrophages (Fig. 3).

These experiments showed that most of the micrococcin injected intravenously as a particulate suspension becomes agglutinated with elements of the blood, and

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EXPLANATION OF PLATES.

FIG. 1.—Particulate micrococcin adhering to the wall of a small vessel after intravenous injection. (Upper L. and Lower R. of vessel wall). 1/7 in. oil-immersion objective.

FIG. 2.—Micrococcin adhering to the walls of healthy small blood vessels invading the clot at the left of the picture. Photograph taken by ultra-violet light, which made the micrococcin fluoresce brilliantly. The rabbit had received 100 mg. of micrococcin intravenously 7 hours before and 100 mg. 1 hour before the photograph was taken. 16 mm. objective.

FIG. 3.—Shows fluorescent macrophages that have taken up micrococcin from material originally deposited intravascularly as in Fig. 2. 16 mm. objective.

FIG. 4.—A chamber in which tuberculous changes are occurring. The black circle at the lower left-hand side of the chamber is a silver pin closing a hole through which tubercle bacilli were introduced 10 days before this photograph was taken. The beginning of necrosis is to be noted to the right of the centre of the field. This is seen more highly magnified in Fig. 5. × 3 approx.

FIG. 5.—A portion of the chamber shown in Fig. 4. An area of increased opacity, possibly due to the necrosis of accumulated cells, can be seen. A compressed vessel can be seen passing through the area. At the time the photograph was taken this vessel still carried a vigorous flow of blood, as can be seen from the fact that the red corpuscles moved during the exposure of 1/50 sec. 16 mm. objective.

FIG. 6.—The same chamber as shown in Fig. 4, 16 days after infection. The necrotic process has extended over nearly the whole of the chamber. The vessels at the periphery on the right are in complete stasis (see Fig. 7). The contents of other vessels in the centre of the field are disintegrating.

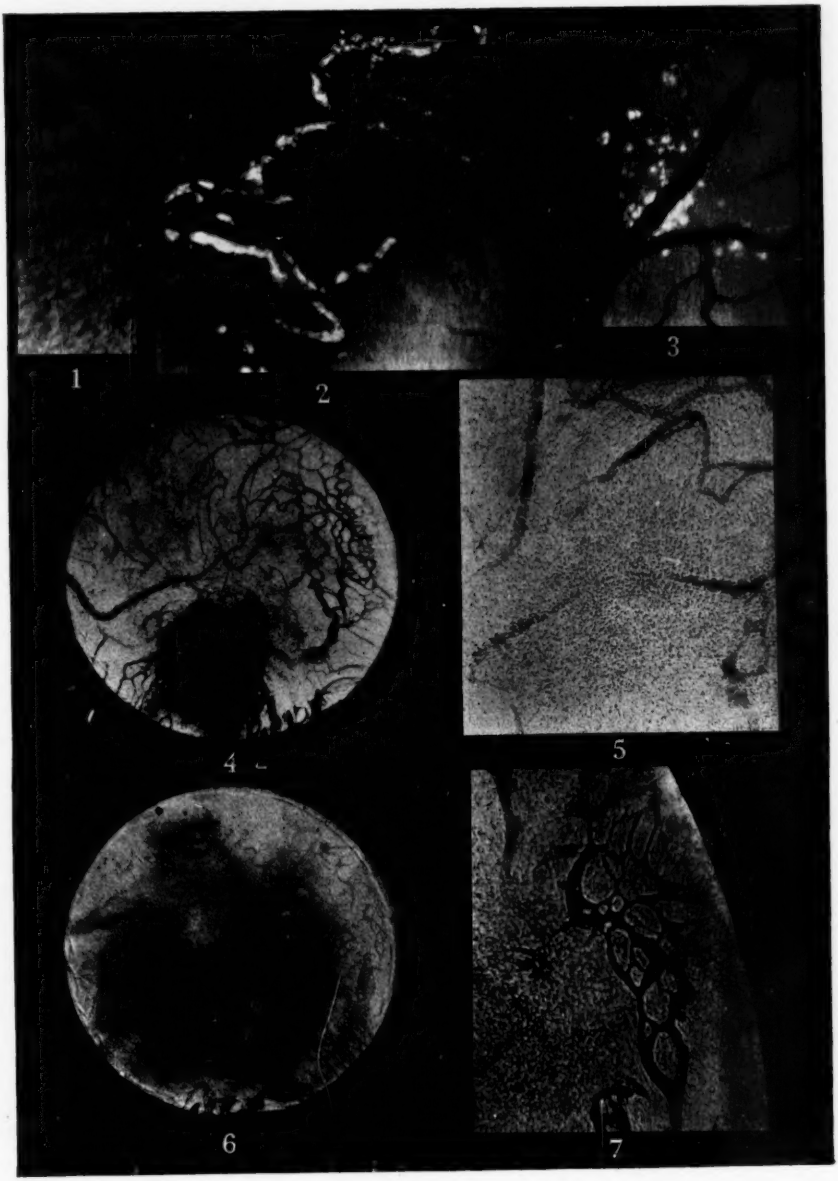
FIG. 7.—A higher magnification of the vessels in stasis shown to the right of Fig. 6. 16 mm. objective.

FIG. 8.—Micrococcin adhering to the walls of the vessels at the periphery of a tubercle. These vessels were still carrying a vigorous circulation. Photograph by ultra-violet light, in which the micrococcin fluoresces brilliantly. 16 mm. objective.

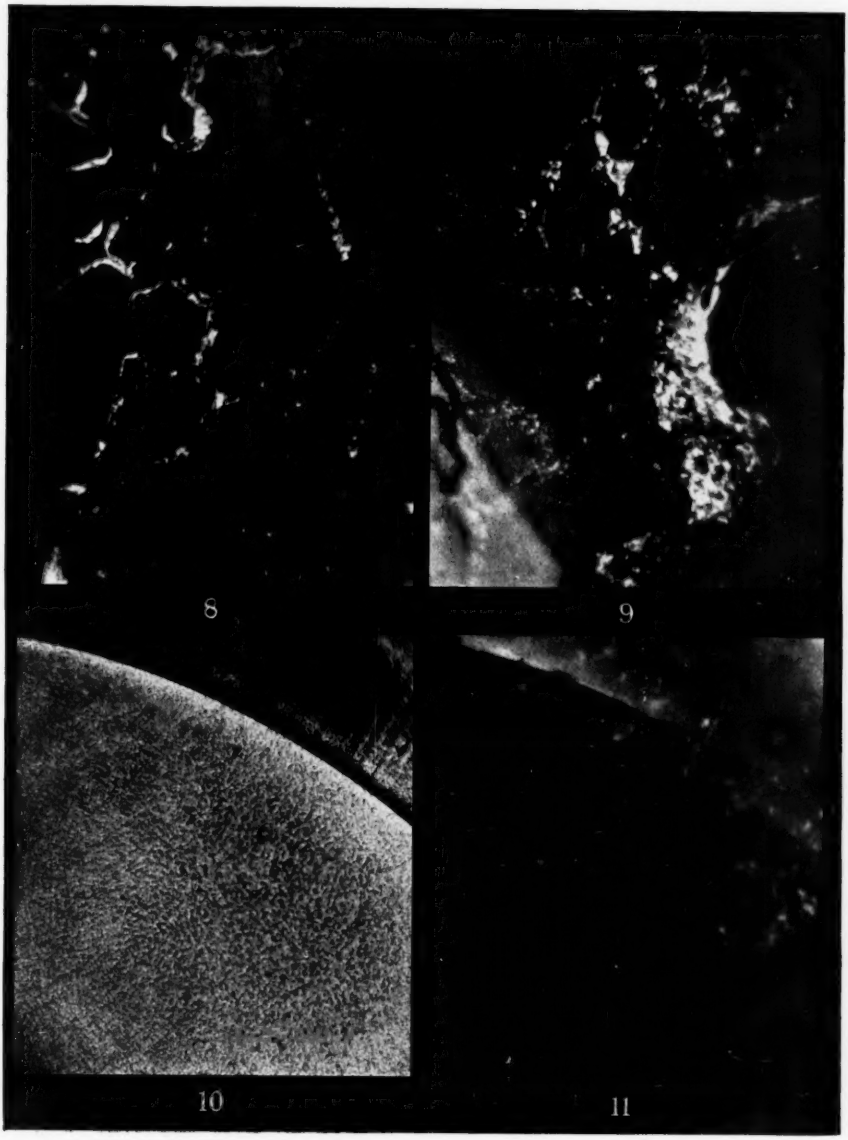
FIG. 9.—Micrococcin in a necrotic tuberculous area. It occupies the position of necrosed vessels, the position of which is not now recognizable by ordinary light. 16 mm. objective.

FIG. 10.—Necrotic tuberculous area at the edge of the table. Micrococcin injected, 5 doses of 200 mg. each. Last injection 8 days before photograph. No structures are visible by ordinary light. 16 mm. objective with green filter.

FIG. 11.—The same field as Fig. 10, seen by ultra-violet light. The outlines of many necrosed blood vessels can be seen because the micrococcin previously adherent to their walls has remained in place.



Sanders, Florey and Wells.



Sanders, Florey and Wells.

that the agglutinated masses adhere to the walls of fine vessels, especially where the endothelium is "sticky," as in the young vessels at the growing edge of newly forming tissue.

In growing tissue some micrococcin becomes extravascular, probably being left behind when the pattern of the vessels alters, but this is only a small proportion of the material originally arrested in the vessels. In more mature vessels the arrested micrococcin seems gradually to be loosened and washed away by the blood stream.

Tuberculous Tissue.

Insertion of tubercle bacilli.

Recently Ebert, Ahern and Bloch (1948) and Ebert and Barclay (1950) have described the tissue reactions occurring when tubercle bacilli are introduced through a removable plug in the back of a transparent chamber in a rabbit's ear.

A similar technique was used in the present work, though chambers of more rigid construction than those of Ebert and his colleagues were employed. A description of these and other chambers, and of the methods of observation, will be given in a later paper.

After the new tissue, which was about 30μ thick, had grown, a silver or stainless steel pin situated eccentrically in the base of the chamber was carefully withdrawn, and the tip dipped in a 6-day-old culture of *Myco. tuberculosis* (bovine type, strain "Branch") in Dubos and Davis's medium. The pin was then replaced, carrying with it tubercle bacilli, which thus came into contact with the tissue. The withdrawal of the pin caused slight damage to the adjacent tissue in the chamber, but this was repaired many days before the development of a tubercle could first be recognized.

In these experiments no attempt was made to follow the earliest cell reactions which no doubt occur soon after the introduction of the bacilli. As observed by Ebert and his colleagues, changes which were obvious under low powers of the microscope began somewhere between the 10th and 15th days after inoculation, and thereafter advanced with some speed. The first change to attract the eye under low magnification is the emergence of opacities in which it is difficult to make out cellular details—indeed it is possible that many of the cells are dead (see Fig. 4 and 5). These areas extend until in a few days the tissue over the whole of the table is necrotic (Fig. 6). During this extension the blood-flow in the vessels at the periphery of the lesion, which are probably maximally dilated, first becomes slowed and usually oscillatory, and then passes into stasis (Fig. 7). In most infected chambers leucocytes adhere in large numbers to the walls of the vessels near the tubercles.

The march of events in the chambers used in this work was much the same as that described by Ebert *et al.* except that there was no increase of free fluid during the growth of the tubercle, nor was dilatation of the blood vessels particularly noticeable. These slight differences were probably due to the layer of tissue being thinner than that in the chambers used by Ebert *et al.* and to its being more rigidly confined. Some chambers used by Sanders, Wells and Wylie (1949, unpublished) warped during their sojourn in the rabbit's ear so that the tissue became thicker, and tubercles growing in such chambers were surrounded by pulsating widely dilated vessels, and much free fluid was present.

Injection of a suspension of micrococcin.

The object of some experiments already performed with micrococcin (Markham, Heatley, Sanders and Florey, 1951 ; Markham, Wells, Heatley and Florey, 1951) was to ascertain whether a particulate antituberculous agent would leave the blood vessels in the neighbourhood of a growing tubercle, and thereafter enter macrophages that were accumulating around the proliferating tubercle bacilli, and further to see whether there was any modification of the tuberculous process by the micrococcin.

In the present experiments micrococcin was injected intravenously as soon as easily recognizable tuberculous lesions were present in the chamber, i.e., at a time when necrosis was probably just beginning. The skin became sensitive to tuberculin at about the time that the lesions started to appear or to extend.

Particulate micrococcin injected intravenously adhered in considerable quantities to the walls of the vessels carrying a circulation in the neighbourhood of the tubercle (Fig. 8), much as in the growing edge of a normal chamber.

In the first experiment a single intravenous injection was given consisting of 100 mg. of micrococcin in 4·5 ml. of diluted serum. Six days after the lodgement of the micrococcin new vessels grew into the tuberculous area in one part, but in 9 days more the chamber was completely necrotic. Two rabbits were subsequently treated with 200 mg. of micrococcin daily for 5 days. Though a considerable amount of micrococcin was arrested in the vessels at the edge of the growing tubercle and subsequently became embedded in the necrotic area, the antibiotic had no apparent effect on the growth of the tubercle. The micrococcin that adhered to the walls of the vessels still carrying a blood stream did not emerge into the tissues in any observable quantity. The course of vessels that had passed into stasis and eventually disintegrated as the necrotic area spread could easily be traced by ultra-violet light, owing to the persistence of the micrococcin that they had contained (Fig. 9, 10 and 11). The micrococcin thus freed into the necrotic part of the tubercle maintained its position and appearance for many days. No doubt the sequence of events just described explains why the carbon injected by Markham and Florey (1951) into tuberculous rabbits entered the caseous centres of tubercles if given soon after infection, but failed to do so when given later.

In all, five chambers were infected in the way described, and three were treated by the intravenous injection of micrococcin. From all chambers tubercle bacilli were recovered in pure culture and were observed in films prepared from the necrotic material.

DISCUSSION.

Observations of living tissue in ear chambers in rabbits have given information concerning the behaviour of intravenously injected particles. It is clear that, although serum does not agglutinate either Indian ink or finely particulate micrococcin *in vitro*, rapid agglomeration of these particles occurs in the circulating blood. This is almost certainly due to the blood platelets. Tocantins (1938) has reviewed the behaviour of blood platelets towards foreign particles such as bacteria injected into the blood stream, and it is clear that much evidence exists to show that platelets and particles tend to adhere together.

In the present experiments platelet masses containing micrococcin adhered

strongly to blood-vessel walls, particularly in places where the endothelium tends to be " sticky," such as a growing edge of new tissue or around a developing tubercle. The adherence of these masses did not precipitate thrombosis in the vessels, and the circulation continued briskly in vessels plastered with such material. Little of this material was transmitted through the vessel walls in uninfected tissue, and it was not possible to see that any was transferred to macrophages in early developing tubercles, even when the vessels surrounding the tubercle contained large amounts of micrococcin.

Even when micrococcin came to lie within the necrotic part of a developing tubercle, through involvement and necrosis of a vessel, there was no observable modification in the development of the tubercle.

SUMMARY.

The behaviour of fine suspensions of carbon and micrococcin injected intravenously has been observed in transparent chambers in the rabbit's ear.

The particles are agglutinated by blood platelets, and the masses adhere firmly to the walls of vessels in such areas as the growing edge of young tissue or the neighbourhood of lesions caused by *Myco. tuberculosis*.

Particles do not readily emerge from the vessels to enter macrophages in the tissues.

No effect on the growth of tubercles was noted following micrococcin injection, even though micrococcin became embedded in the central necrotic portion of developing tubercles.

We are indebted to the Medical Research Council for grants for technical assistance and materials; to Mr. B. K. Kelly and his staff, of the Antibiotics Research Station of the Medical Research Council, for the fermentation and partial preparation of the micrococcin used in this work, and to Dr. N. G. Heatley for the preparation of the micrococcin suspensions. We are grateful to Mr. J. Kent for his very skilful technical assistance and for his expert care of the animals, to Mr. J. Causton and Mr. A. A. Argyle for their assistance, and to Mr. S. W. Bush for making the transparent chambers. We are indebted to Mr. C. A. Graham for help in the preparation of the photographs, and to Miss W. M. Poynton for help in the preparation of the paper.

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THE EFFECTS OF A FOLIC ACID ANTAGONIST (AMINOPTERIN) ON ALBINO RATS: A STUDY IN THE PATHOGENESIS OF SPRUE.*

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RECENT investigations of folic acid and its antagonists yielded many interesting but as yet not well-coordinated conclusions. Of particular relevance to this report are the observations on the effects of folic acid in macrocytic anaemias, notably sprue (Manson-Bahr, 1946; Speis, Lopez, Milanes and Aramburu, 1947), and of the folic acid antagonists in cases of leukaemia (Farber, 1949) and experimental tumours (Woll, 1948*a*).

In a study of chicks fed a synthetic folic acid-free diet, or an adequate diet supplemented with a folic acid antagonist (4-amino-pteroylaspartic acid), constant alterations were observed in the bone-marrow and gastro-intestinal tract (Woll, 1948*b*). These were identical in the diet-deficient and antagonist-treated animals. The marrow showed a transient reduction in the mature red and white blood cells and maturation arrest of both red and white elements, with an increase in the size of the stem cells. The process progressed rapidly to a total aplasia. In the gastro-intestinal tract there was, initially, marked hydropic swelling of gland cells of the small bowel and colon with cystic dilatation of the gland lumen. A comparable cytologic change was seen in the oesophageal mucosa. The process advanced to a marked atrophy and early scarring. The stomach escaped this. In order to verify the above observations on a different species of animal and with a more potent antagonist, essentially similar experiments, using albino rats and 4-amino-pteroylglutamic acid (aminopterin), were performed. The findings are here reported.

MATERIALS AND METHODS.

Seventy albino rats weighing 110 to 130 g. were divided into the following three groups:

1. Control: commercial food and water *ad lib.* (15 animals).
2. Diet as above, plus a daily dose of 25 μg. aminopterin administered orally in aqueous solution by catheter (35 animals).
3. Diet as above, plus a single oral dose of 100 μg. aminopterin given by catheter (20 animals).

In order to compare the effects of starvation and a water-free regime on the same colony of animals, an additional fourth group of 10 rats was kept without

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food and a fifth group of 10 rats without water. The cages for all groups were of a type which greatly reduced the chance of the rats eating their excreta.

The animals were killed with ether according to the following schedule: Group 1 (control): one to three animals at 3-day intervals from the 3rd to the 24th day of the experiment. Group 2 (daily dose of aminopterin): two to three animals were killed daily from the 2nd to the 24th day. Group 3 (single dose of aminopterin): two to three animals at 3- to 9-hour intervals from the 1st to the 3rd day of the experiment. The rats on the starvation and water-free regimes were killed in groups of 2 or 3 every 3 days for the first 10 days of the experiment. Autopsies were performed on all immediately after death. Representative blocks of tissue were fixed in formaldehyde or Zenker-formaldehyde solutions. Paraffin sections were prepared and stained with haematoxylin and eosin. In some instances special connective tissue, fat, and glycogen stains were made on suitably fixed material. In all cases direct smears of the femoral marrow were prepared, fixed in methyl alcohol and stained with haematoxylin and eosin or Wright's stain. Sections of intact femoral marrow were also prepared.

RESULTS.

Ante-mortem and autopsy gross findings.—Ante-mortem the following changes were of progressive severity for the duration of the experiment. The antagonist-treated animals were markedly malnourished and dehydrated. The fur was rough. There was no alopecia. Often the rats assumed a hunched position. There was no paralysis or evidence of muscle incoordination. The snout, paws, eyelids and tails were stained dark brown. This pigment gave a deep red glow under ultra-violet light. Diarrhoea, progressively more severe, became haemorrhagic. It was pronounced in animals which received the single large dose of the antagonist. In no case was there bleeding of other than gastro-intestinal origin.

At autopsy the chief findings were: Severe anaemia, dehydration of the muscles and subcutaneous tissues, greatly shrunken spleen, thymus, and lymph nodes. The bowel was either contracted, pale and opaque, with a swollen mucosal coat, or markedly distended with a foul-smelling pale grey or dark brown watery fluid. There were large ulcers of the small and large bowel. In all cases the stomach failed to show appreciable deviations from normal.

The femoral marrow was scant, deep red, and terminally pale brown, viscid. The adrenals were slightly larger than normal. They were otherwise not remarkable.

Microscopic examination.—The earliest changes were noted in the bone marrow. These were seen on the second day of the experiment. In the animals which received the large dose of antagonist, the alterations were observed in 24 hours. At first there was a sharp decrease in the number of mature granulocytes. This was followed by an absolute increase in the number of stem cells and decrease in normoblasts. The stem cells increased in diameter 40–60 per cent as measured by a micrometer under oil immersion. Their nuclear detail became indistinct rather than more delicate as in the megaloblasts of pernicious anaemia. The cytoplasm also increased in amount and stained more lightly than normal. Finally most of the normal cellular elements of the marrow disap-

peared, leaving an occasional erythroblast, a few lymphocytes and prominent reticulo-endothelial cells (Fig. 1, 2, 3).

The spleen, lymph nodes and thymus showed progressive depletion of the mature elements. There was no actual increase in the reticulo-endothelial cells. The contraction of these organs brought these cells into greater prominence. Early in the experiment both the thymus and mesenteric lymph nodes showed numerous irregularly shaped basophilic particles in the sinuses. Whether these represented fragments of chromatin is not certain. Phagocytosis was at no time prominent (Fig. 4, 5).

In the gastro-intestinal tract there was, initially, a marked swelling of the cytoplasm and nucleus of the cells of the glands, followed by severe disorganization of the normal cellular configuration, cystic dilatation of the gland lumen, and then severe atrophy of all epithelial elements. Cells in mitosis were seen only slightly less frequently than in the control animals. This was followed by severe ulceration, acute purulent exudate, haemorrhages, and early evidence of fibrosis (Fig. 6, 7, 8). Of interest was the finding that the Paneth cells escaped, until very late, the above-described degenerative changes.

The lesion in the gastro-intestinal tract followed quite consistently the following sequence: The first parts involved were the ileum and duodenum, followed by the colon and then the oesophagus (Fig. 9, 10). Later and rarely the tongue also was involved. The stomach always escaped this process entirely.

No appreciable departures from normal were seen in the other viscera. The adrenal, while heavier than usual, showed no appreciable morphologic alterations. In the brain the cortical neurons in the anterior half of the cerebral hemispheres appeared larger than normal and stained more lightly. However, at no time was there evidence of reactive changes. The skeletal system, peripheral nerves, cord and the pituitary glands were not examined with sufficient regularity to warrant valid conclusions. The animals on the control, starvation and water-free regimes failed to show any of the above-described lesions. The

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EXPLANATION OF PLATES.

FIG. 1.—Normal rat femoral marrow. Haematoxylin and eosin. × 1000 approx.

FIG. 2.—Femoral marrow of a rat which received aminopterin for 7 days. Note the swelling of the immature cells, loss of chromatin detail and reduction in the developed blood cells, especially granulocytes. Haematoxylin and eosin. × 650 approx.

FIG. 3.—Rat femoral marrow after 20 days on an aminopterin-supplemented diet. Note the severe pancytopenia, increased prominence of the reticulo-endothelial elements and the myxomatous character of the stroma. Haematoxylin and eosin. × 650 approx.

FIG. 4.—Normal rat thymus. The cortex occupies most of the field. Haematoxylin and eosin. × 300.

FIG. 5.—Rat thymus after 7 days of aminopterin-supplemented diet. Note the dark particles of varying size and shape in the cortex. Haematoxylin and eosin. × 300.

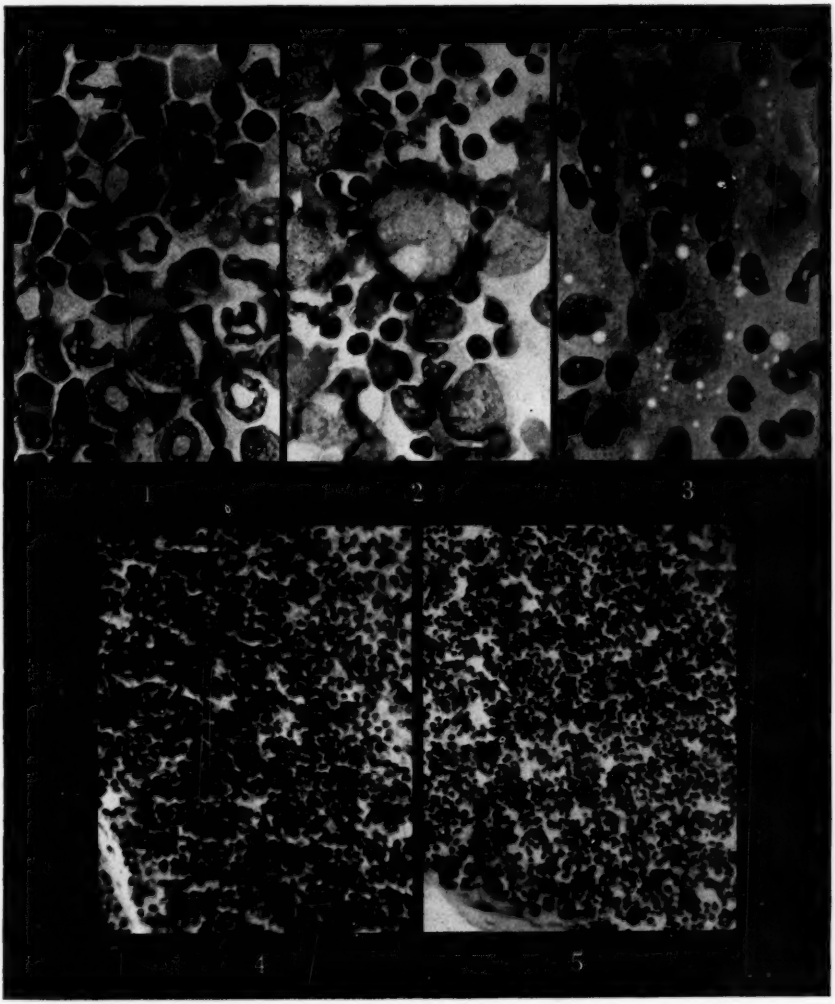
FIG. 6.—Normal rat duodenum. Haematoxylin and eosin. × 300.

FIG. 7.—Rat duodenum after 7 days of aminopterin-supplemented diet. There is marked nuclear swelling, a prominent nucleolus, disturbed cellular configuration and swelling of the cytoplasm. Haematoxylin and eosin. × 300.

FIG. 8.—Rat duodenum after 15 days of an aminopterin-supplemented diet. Note the severe atrophy, cystic dilatation and fibrosis. Haematoxylin and eosin. × 300.

FIG. 9.—Normal rat oesophageal mucosa. Haematoxylin and eosin. × 300.

FIG. 10.—Rat oesophageal mucosa after 22 days of an aminopterin-supplemented diet. The sequences of maturation and keratinization are incomplete. Cellular outline and chromatin detail are lost. Activity in the basal and intermediate layers is apparently reduced. Haematoxylin and eosin. × 300.



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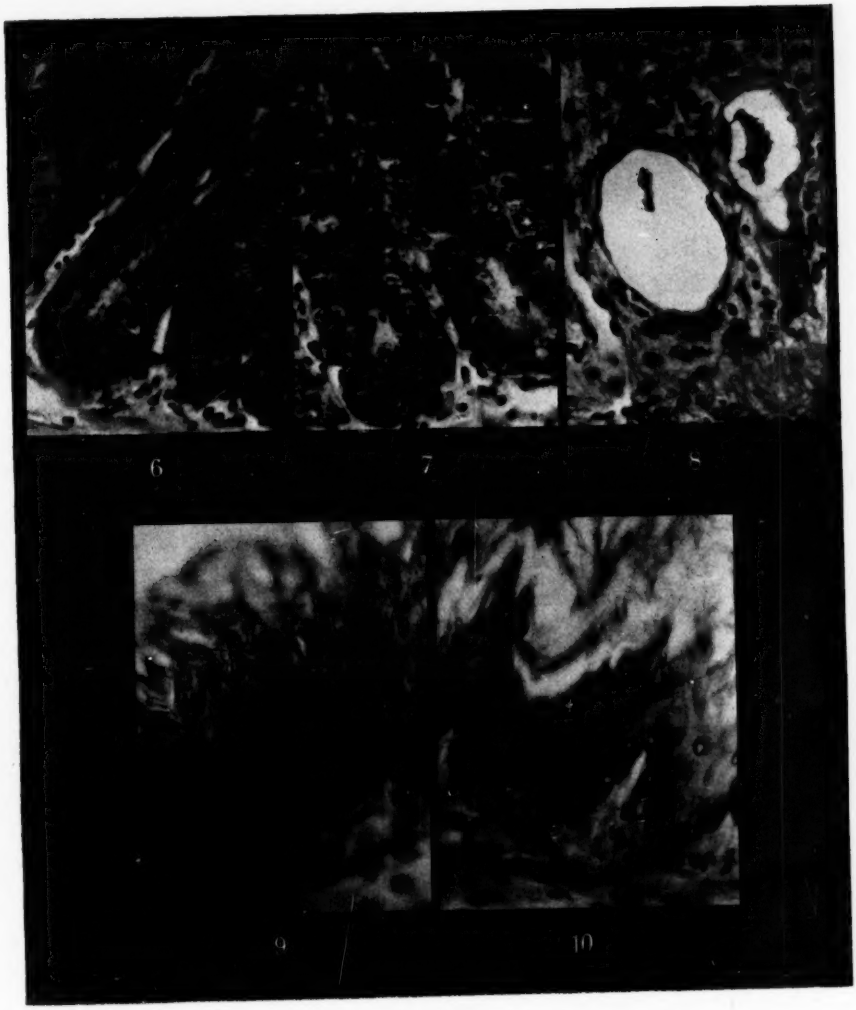

Woll and Oleson.

starved and dehydrated animals showed general changes of inanition characterized by generalized gross and microscopic reduction in the size of organs and parenchymal cells.

DISCUSSION.

The specific morphologic pathology of sprue, not related to pancreatic insufficiency, is fragmentary and conflicting. The lesions of the bowel described in this condition are quite similar to those seen in the above experiments. It is possible that the study of experimental folic acid deficiency, either diet- or antagonist-induced, may contribute to the knowledge of the pathogenesis of this disease.

SUMMARY.

Albino rats, fed a folic acid antagonist, were killed at frequent intervals, autopsied and the tissues examined microscopically. The findings were compared with those in control, food- and water-deprived animals.

The chief alterations were: (*a*) A pancytopenia and maturation arrest of the red and white blood cells at the level of the stem cell, with macrocytosis; this progressed to total aplasia; (*b*) atrophy of the gastro-intestinal tract, which had a specific pattern and did not involve the stomach.

The changes were essentially similar to those seen in chicks made folic acid-deficient by diet or antagonist.

The possible implications in relation to sprue are pointed out.

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TWO MECHANISMS WHICH INCREASE *IN VIVO* THE LIVER TRYPTOPHAN PEROXIDASE ACTIVITY: SPECIFIC ENZYME ADAPTATION AND STIMULATION OF THE PITUITARY-ADRENAL SYSTEM.

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THE activity of the tryptophan peroxidase system (Knox and Mehler, 1950) in the livers of rats is greatly increased within a few hours of administration of tryptophan or of certain other compounds (Knox and Mehler, 1951). In the present paper it will be shown that this phenomenon is due to two independent mechanisms: (1) The increase in the enzyme activity following the administration of the substrate tryptophan is due to a mechanism resembling that of enzyme adaptation in micro-organisms. (2) The increase in the enzyme activity after administration of certain other substances which are not substrates of the enzyme system can be considered as a physiological adjustment characteristic of animals (Knox and Mehler, 1951), and is due to stimulation of the pituitary-adrenal system. The two mechanisms by which the enzyme activity can be increased, one adaptive and one hormonal, are of additional interest in revealing a potential means of regulating metabolism, not by affecting the enzyme reaction itself, but by altering the amount of the enzyme.

METHODS.

Hooded and albino rats of both sexes, weighing 180 to 220 g., were used. No strain or sex differences in enzyme activity were noted. The adrenalectomized animals were operated upon under ether anaesthesia, since nembutal treatment was found to depress the tryptophan peroxidase activity. They were then given 1 per cent NaCl as drinking water, and used on the third post-operative day. The completeness of adrenalectomy was verified by gross post-mortem examination.

The various compounds to be tested were injected intraperitoneally into both intact and adrenalectomized rats, as solutions or suspensions in 4 ml. water. The animals were killed five hours later, by which time full development of the enzyme increase had occurred (Knox and Mehler, 1951). Untreated animals were included in each group studied. The livers were removed promptly after death, cooled in ice, homogenized for 2 min. in a Waring Blendor with 8 volumes of cold 0·9 per cent KCl, and each immediately assayed.

Enzyme assay.

Two concentrations of each homogenate, 1·0 and 2·0 ml. (0·5 and 1·0 ml. of highly active homogenates) in 4·0 ml. total volume were assayed. Additions

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were 0·5 ml. 0·2 M phosphate, pH 7·0, and 0·3 ml. 0·03 M L-tryptophan (omitted from the blank run with each concentration). These mixtures were incubated aërobically with shaking at 38° for 1 hr., and the kynurenine formation determined, after Zn acetate-NaOH de-proteinization, by the increased absorption at 360 mμ. in a Beckman spectrophotometer.

Under these conditions L-tryptophan is oxidized to formyl-kynurenine by the two-step coupled oxidation reaction of the tryptophan peroxidase-oxidase system. The formyl-kynurenine is in turn hydrolyzed to kynurenine by the larger excess of formylase present in the liver homogenate. No added source of peroxide was necessary for the tryptophan peroxidase in these experiments, since the endogenous production of peroxide during the first two hours after preparation was adequate for full activity. The kynurenine formation was proportional to time up to several hours, and to the amount of the tryptophan peroxidase-oxidase system (Knox and Mehler, 1950). The activities determined with the two enzyme concentrations in each case were averaged, although the higher enzyme concentrations of the most active preparations were about 10 per cent less active than expected. This deficit in kynurenine accumulation was traced to a small activity of kynureninase (*cf.* Dalgliesh, Knox and Neuberger, 1951) which occurred at the higher kynurenine concentrations. This small further reaction of kynurenine tended to diminish rather than to exaggerate the increases in the tryptophan enzyme activity, so the error incurred has therefore been ignored.

RESULTS.

Action of other compounds compared to tryptophan.

The compounds which have been tested for their ability to increase substantially the tryptophan peroxidase activity in rat livers within five hours, including those previously reported on (Knox and Mehler, 1951) are listed in Table I, and arranged according to their effects on the enzyme level. The effective com-

TABLE I.—*The Levels of Activity of the Tryptophan Peroxidase-Oxidase System in Rat Liver after Administration of Various Compounds.*

Enzyme activity in liver after treatment.		
Normal.	Moderately increased. (approx. 2-fold).	Greatly increase (approx. 10-fold).
Na *L*-glutamate (6)	*DL*-phenylalanine (2)	*DL*-tryptophan* (11)
DL-alanine (2)	*L*-tyrosine (2)	*L*-tryptophan, 1 m.mol. (3)
Na acetate (2)	Na anthranilate (2)	
Sucrose (2)	Na gentisate (2)	
Ammonium chloride (2) (orally)	*L*-kynurenine* (1)	
	L-histidine-HCl* (12)	
	Histamine acid phosphate 0·005 m.mol. (4)	
	Epinephrine 0·001 m.mol. (6)	

* Levels produced by 2 m.mols of compounds so marked approximate the levels from 1 m.mol *L*-tryptophan.

L-tryptophan, epinephrine and histidine were given in the doses indicated, the other compounds in dose of 2 m.mols, by intraperitoneal injection to normal rats five hours before death. The number of animals given each compound is indicated in parentheses.

pounds other than tryptophan are: (1) inactive as substrates of the tryptophan peroxidase, (2) not necessarily related to each other or to tryptophan, (3) produce generally only double the normal level of enzyme in contrast with the ten-fold increases often produced by tryptophan, and (4) show wide differences in at least two cases in the dose necessary to produce the effect. A 1 m.mol dose of L-tryptophan, wehther it was given as such or as 2 m.mols of DL-tryptophan, increased the enzyme maximally. At least twice this dose of L-histidine or L-kynurenine (i.e., 2 m.mols of the L-isomer) was necessary to produce enzyme levels approaching those obtained with 1 m.mol of L-tryptophan. These compounds were accordingly classed as moderately effective. The other effective compounds only about doubled the normal enzyme activity. Adrenaline and histamine, however, produced this moderate increase with only about one-thousandth the dose necessary with the other compounds. These dosage differences, and the other differences listed above, suggested that the effective non-tryptophan compounds increased the enzyme by a separate mechanism from that of tryptophan itself.

Non-substrate compounds act through the pituitary-adrenal system.

A common mechanism by which the variety of non-substrate compounds could produce these similar increases was thought to be the relatively non-specific stress reaction. This reaction of the pituitary-adrenal system is well known to be stimulated by a wide variety of agents. Such stimulation could be expected simply from the large doses of the more insoluble compounds in themselves. Hyper-irritability and even coma were occasionally observed in the treated animals. Even some of the compounds listed in Table I as ineffective did irregularly cause moderate increases in enzyme activity when given to rabbits in massive doses. The stress reaction was specifically implicated by the increase in enzyme activity produced with small doses of adrenalin or histamine. Such doses of these two compounds to rats are known to cause adrenocorticotrophic hormone (ACTH) release and adrenal stimulation, reproducing the stress reaction (Long and Fry, 1945; Sayers and Sayers, 1948).

The role of the pituitary-adrenal system in producing the increased enzyme activity due to the moderately effective compounds was confirmed by studies of adrenalectomized animals (Table II). The levels of enzyme activity found in a series of untreated and treated intact rats have been tabulated in the upper half of the Table. The treated rats were given the most active non-tryptophan compounds: adrenaline and histamine, which act in very small doses, and histidine, which produces levels of enzyme activity approaching those due to tryptophan itself. A highly significant increase in the enzyme activity above the levels found in untreated animals was produced by each compound. In the lower half of Table II are given the levels of enzyme activity found after administration of these compounds to adrenalectomized rats. (Histamine was not tested in adrenalectomized rats). Some small effects may be noted, such as the possibly significant decrease in the enzyme activity in untreated animals due to adrenalectomy itself. But the most striking effect was the marked decrease or elimination after adrenalectomy of the usual enzyme responses to adrenaline and histidine. This result establishes the part played by the adrenal glands in increasing the liver tryptophan oxidizing activity after doses of the moderately effective, non-tryptophan compounds.

TABLE II.—*Comparison of the Activity of the Liver Tryptophan Peroxidase System Produced by Compounds Administered to Normal and to Adrenalectomized Rats.*

Enzyme activity (μmols kynurenine formed/g./hr.).

Compound given.	No treatment.	Epinephrine (0·2 mg.).	Histamine acid phosphate (2 mg.).	*L*-histidine HCl (0·31 g.).	*DL*-tryptophan (0·3 g.).
Normal rats	7·5	16·9	13·0	22·5	24·1
	8·4	23·2	15·6	36·4	27·0
	8·5	33·2	23·3	37·6	31·9
	8·4	27·2	..	35·9	51·9
	12·4	18·7	..	38·5	73·0
	10·6	25·6	..	44·2	96·2
	..	..	..	48·1	..
	..	..	..	50·2	..
Ave. ± σ	9·3±1·8	24·1±5·9	17·3±5·6	39·2±8·6	50·7±29·0
Adrenalectomized rats	6·3	6·4	..	6·4	25·5
	4·9	9·1	..	7·2	27·3
	7·8	3·2	..	9·2	42·0
	5·4	6·5	..	9·9	40·2
	3·7	15·7	..	10·3	35·9
	..	10·6	..	14·4	62·2
Ave. ± σ	5·6±1·5	8·6±4·5		9·6±2·8	38·9±13·2

The rats were killed for enzyme assay five hours after intraperitoneal administration of the stated doses of the test compounds. The adrenalectomized animals given tryptophan died spontaneously in about 3½ hours, at which time the livers were promptly assayed. The enzyme activity given for each animal, in μmols kynurenine formed/g. dry wt. of liver/hr., is the mean of two determinations with different concentrations of each liver homogenate.

Action of tryptophan.

By the same type of experiment tryptophan was shown to act in a way different from that which involves the adrenal glands. The levels of enzyme induced by tryptophan administration to both intact and adrenalectomized rats are also given in Table II. The variable absorption of this insoluble amino acid within the time allotted produced a wide range of activities. Despite this variation, it can be seen that tryptophan produces large increases in the activity in intact rats and substantially as large increases also in adrenalectomized rats. The slightly lower levels in the adrenalectomized animals can be attributed to their spontaneous death about 4 hours after the dose of tryptophan, before the 5 to 6 hours necessary for full development of the enzyme increase. This exceptional toxicity of tryptophan to adrenalectomized animals is unexplained, although it does not prevent the adrenalectomized animals from developing amounts of enzyme after tryptophan administration comparable to those developed by intact animals. This response of the adrenalectomized animals to tryptophan, but not to the other compounds, clearly indicates the presence in animals of a specific mechanism which increases enzymic activity only after treatment with the substrate itself.

DISCUSSION.

The principal difference between the process in animals which results in increased tryptophan peroxidase and that in micro-organisms known as enzyme adaptation was previously pointed out to be the effectiveness of certain non-substrate compounds in increasing the animal enzyme (Knox and Mehler, 1951). These other compounds have now been shown to act separately from tryptophan, and the specific increases of the tryptophan peroxidase after tryptophan administration, as it occurs in adrenalectomized rats, can now be compared to the general process of enzyme adaptation.

Definition of enzyme adaptation in micro-organisms.

From various descriptions of the phenomenon (Dubos, 1940; Monod, 1947; Spiegelman, 1950) three characteristics have been selected which provide a conservative definition of enzyme adaptation. It should be emphasized that even in the field of microbiology these three characteristics have not necessarily all been demonstrated in each case accepted as enzyme adaptation.

1. A valid increase in the activity of a particular enzyme: Apparent increases, due to permeability changes or co-factor augmentation for example, are excluded by demonstration of the increased activity also in cell-free preparations and under the optimal conditions for the reaction.

2. The increased activity is produced by treatment of the cells with the enzyme's substrate or a very closely related compound. The mutual specificity of the compound inducing the adaptation and the enzyme it calls forth, a relationship like that of enzymes and their substrates (Mirick, 1943), is a positive characteristic of the phenomenon.

3. The increased activity is not referable to an increase in the mass of cells or to the selection of special cell types. The cells must be able to carry out synthetic reactions to adapt (Spiegelman, Kamen and Sussman, 1948), but need not grow (Stephenson and Stickland, 1933; Pollock, 1946; Brandt, Freeman and Svenson, 1951).

Characteristics of the increase in the tryptophan peroxidase.

Validity of the increase.—The high activity of the tryptophan peroxidase after treatment was previously demonstrated in liver slices and in cell-free preparations under the optimal conditions of the assay. The increased activity could not be attributed to permeability changes, provision of co-factors, reversal of inhibition, etc., and was accepted as a valid increase in activity, probably due to an actual increase in the enzyme concentration (Knox and Mehler, 1951).

Specific relation of enzyme to adapting compound.—The production of the increased activity by the substrate of the enzyme, tryptophan, and not by unrelated compounds, has here been demonstrated in adrenalectomized animals. In intact animals both this adaptive process and a second process involving the hormonal system can occur.

Independence from growth.—The increase in the tryptophan enzyme in liver by growth of a particular cell-type rich in this enzyme is reasonably precluded by the ten-fold magnitude of the activity change occurring within six hours. The generation time (for doubling) of fibroblasts in tissue cultures is about 24

to 48 hours (Fischer, 1946), and must be even longer for more highly differentiated cells *in vivo*. The rapid decrease to normal activity within eighteen hours as the given tryptophan is utilized (Knox and Mehler, 1951) is additional evidence that the change is not of the relatively permanent kind resulting from growth of new cells. Similar rapid losses of adaptive enzymes upon withdrawal from the substrates commonly occur with micro-organisms (Dubos, 1940; Spiegelman, 1946; Mirick, 1943). Moreover, isolated liver slices from untreated animals, incubated in oxygen, glucose and tryptophan, did not increase their tryptophan oxidizing activity. The synthetic abilities of liver are relatively poor under these conditions, and the failure to adapt, may be compared to the similar inability of dinitrophenol-treated yeast cells to adapt, in each case the adaptation being prevented by the lack of synthetic reactions (Spiegelman *et al.*, 1948).

From these considerations the increased tryptophan peroxidase produced by the administration of tryptophan can be accepted as an example of enzyme adaptation occurring in animals.

General adaptive mechanisms in animals.

There is a wealth of phenomena in animals which possibly includes other examples of enzyme adaptation (see also Bodansky, 1948). Among these are variations in the activities of arginase (Lightbody and Kleinman, 1939), β-glucuronidase (Fishman, 1940), pancreatic amylase and trypsin (Grossman, Greengard and Ivy, 1942), kidney glutaminase (Davies and Yudkin, 1951), and succinic dehydrogenase (Shipley, Meyer, Copenharer and McShan, 1950). In none of these cases have the validity of the increased activity, the close relationship between the compound and the adapting enzyme, and the independence of the increase from cell growth or replacement all been demonstrated. None can yet be accepted as enzyme adaptation on the basis of the criteria used above. Further work on the arginase and β-glucuronidase has indeed shown these changes in activities to be referable to other processes (Miller, 1950; Kerr, Campbell and Levvy, 1949). But with the identification of one example of enzyme adaptation in animals, the likelihood is increased that other similar changes are due to the same cause.

It is necessary, however, to distinguish between those enzymic changes due to enzyme adaptation, as a particular process, and those due to the other mechanisms which contribute to the general adaptability of living systems (growth, heredity and the varied processes accounting for the responsiveness of protoplasm). The criteria of enzyme adaptation used here are purposely conservative in order to minimize the confusion of this poorly understood process with the others. Among such other processes from which enzyme adaptation must be distinguished in animals is the action of hormones.

The hormonal mechanism increasing the tryptophan peroxidase.

The second mechanism which produces increased activity of the tryptophan enzyme was identified as an effect of the pituitary-adrenal system by its initiation with small doses of adrenalin or histamine, or with larger doses of possibly injurious compounds, and by its elimination upon adrenalectomy. ACTH is released from the pituitary by certain specific and many non-specific agents, including adrenalin and histamine, with consequent stimulation of the adrenal cortex and

production of the so-called stress or alarm reaction. The enzyme would then appear to increase in response to the adrenal hormones, a process distinct from its adaptive increase in response to the substrate. The effective compounds other than adrenalin and histamine may act by causing ACTH release (*cf.* Blanchard, Dearborn and Marshall, 1951), or only after their metabolic conversion to active amines (e.g. histidine to histamine).

The variety of effects following adrenal cortical stimulation is also called the general adaptation syndrome (Selye, 1946), which despite the similarity of names should not be confused with enzyme adaptation. One burden of the present work is to show that these are separate processes, which in this case share a common ultimate effect—an increase in the amount of the same enzyme. It is of interest that the increase in the tryptophan peroxidase develops in a few hours, and earlier than other objective effects of the stress reaction or of ACTH administration (White, 1949).

Changes in enzyme concentration as a process regulating metabolism.

The marked fitness between the stimulus and the response in enzyme adaptation suggests the possible function of this process. Enzyme adaptation can proportion the amount of an enzyme to the amount of its substrate, and in this way adjust the metabolism for the work in hand. The metabolism of L-tryptophan in animals, leading ultimately to the formation of nicotinic acid, is initiated by the tryptophan peroxidase and may be controlled by the amount of this enzyme. Since a very large change in the enzyme was produced by supplementary tryptophan equal to about twice the daily intake, a metabolically significant change could also be expected from the small fluctuations of the dietary tryptophan.

The metabolic regulatory mechanisms studied in animals up to the present have been largely hormonal. In general, the hormones have rarely been found to affect appropriate cell-free enzyme reactions directly, although in intact animals they obviously affect the metabolism utilizing these reactions. The separate processes of enzyme adaptation and of hormonal action, in the present study, both had the effect of increasing the amount of an enzyme *in vivo*. The production of a potential increase in metabolism by increasing the amount of enzyme, but without affecting the catalytic activity of a given amount of enzyme may therefore be a general means of metabolic regulation. Action of hormones by changing the amounts of enzymes concerned would account for the modifications of enzyme concentrations characteristic of many states of endocrine disbalance (Dempsey, 1946). And since changes in the amounts of the enzymes can only be produced by certain synthetic functions of the cell, the action of the hormones would understandably be not readily demonstrated in cell-free preparations.

SUMMARY.

Two mechanisms producing an increased amount of the liver tryptophan peroxidase in animals are described: the first produces large increases and acts only in response to the administration of the substrate, tryptophan; the second produces only moderate enzyme increases and acts in response to a variety of compounds, including adrenalin and histamine. These mechanisms both act in the normal animal, but adrenalectomy eliminates the second, the less specific and less effective process. The first mechanism has been identified as that of

enzyme adaptation, previously known only in micro-organisms, and the second has been identified as the stress reaction, acting through the adrenal glands.

The criteria of enzyme adaptation in micro-organisms on which the identification of this process in animals is based are: (1) a valid increase in enzyme activity; (2) which is produced by treatment with the substrate of the enzyme; and (3) which occurs independently of growth or selection of cells. It is also necessary in mammals to differentiate other processes which can alter enzyme activity, like the hormonal effects of the stress reaction acting through the adrenal glands in the present example.

These results suggest that alterations in the quantities of enzymes may be a general means by which metabolism is regulated.

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